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Development of an autologous dendritic cell vaccine for the treatment of malignant melanoma and high grade gliomas

by

Robert Gawley Kerr ©

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of the requirements for the degree of Doctor of Philosophy

in

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Abstract:

Gliomas and melanomas are highly malignant tumours both of neuroectodermal origin that despite treatment with currently available surgical techniques, radiotherapy, and chemotherapeutics have very poor prognosis. We review here the concepts of immunotherapy for the treatment of these tumours and focus on the application of dendritic cell (DC) mediated strategies. In Chapter 2 we present our *in vitro* studies of the development of an autologous DCs. We demonstrate the ability to consistently generate DCs from circulating peripheral monocytes and subsequently prime them with peptides derived from early explant tumour cells. We also confirmed that autologous and HLA-A2 matched cytotoxic T-lymphocytes (CTLs) could be cross-primed/activated by tumour peptide primed DCs as evidenced by *in vitro* CTL clonal expansion, interferon gamma secretion and ability to kill target tumour cells.

In Chapter 3 we present progress of a Health Canada approved autologous DC vaccination trial combined with immunoadjuvant therapy for the first four patients treated. Patients harboring stage III or IV melanoma who had progression despite conventional treatment were vaccinated with autologous DCs primed with peptides derived from autologous early explant tumour cultures. We demonstrate that this treatment was safe, resulted in robust CTL responses, and suggested efficacy with respect to patient survival.

Many tumours have evolved complex methods of avoiding immune system recognition. The cation independent- mannose 6 phosphate receptor (CI-M6PR) is an important mediator of apoptosis induced by granzyme B (GrB) released from CTLs. In chapter 4 we present evaluation of melanoma and glioma cells for surface CI-M6PR expression and identify for the first time in a glioma tumour an abnormality of the CI-M6PR that is associated with membrane accumulation and functional failure to internalize GrB and complete resistance to GrB or CTL mediated killing. This is a finding that has important implications for immune based therapies.

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Table of Contents

Table of contents
List of Tables
List of Figures
List of Abbreviations

Chapter 1. Treatment of Malignant Melanoma and High Grade Gliomas 1-39

- Introduction 2-5
- Current Surgical, Radiation, and Chemotherapy Treatment Strategies 5-10
- Introduction to Immunotherapy for Malignant Melanoma and High Grade Gliomas 10-22
- References 22-39

Chapter 2. Human Dendritic Cells Primed With Glioma or Malignant Melanoma Naturally Processed Tumour Peptides Induce Cytotoxic Anti-tumour Response *In Vitro* 40-70

- Introduction 41-44
- Methods 44-54
- Results 54-67
- Discussion 67-68
- References 69-70

Chapter 3. Progress Report of a Phase I Autologous Dendritic Cell Vaccine Trial for the Treatment of Patients With Malignant Melanoma 71-143

- Introduction 72-77
- Methods 76-92

- Results 92-121
- Discussion 122-132
- References 134-143

Chapter 4. Evaluation of the Role the Cation Independent Mannose 6 Phosphate Receptor May Play in Avoidance of Cytotoxic T-Cell Induced Apoptosis by Glioma and Melanoma Tumour Cell Lines 144-193

- Introduction 145-152
- Methods 153-161
- Results 161-176
- Discussion 176-181
- References 182-193

List of Tables

Table 2-1. Naturally Processed Tumour Peptide Preparations 55

Table 3-1. Serum Immunoglobulin Levels 109

Table 3-2. Pathology Evaluation of Immune Cell Infiltration 111

Table 3-3. DC Trial Patient Staging and Treatment 120

Table 3-4. DC Trial Patient Interventions and Progress 121

Table 4-1. Cell Lines Evaluated by FACS 154

List of Figures

Figure 2-1 Microscopy Evaluation of Dendritic Cells 57

Figure 2-2. Dendritic Cell Surface Expression of Markers of Maturation 58

Figure 2-3 Comparison of Two Methods of DC Maturation 59

Figure 2-4. Flow Chart of DC Preparation and CTL Cross-Priming 60

Figure 2-5 Clonal Expansion of Autologous CTLs 62

Figure 2-6 IFNγ Release with Repeated CTL Repriming with HLA-A2 Matched Peptide Pulsed DCs. 63

Figure 2-7 IFNγ Release by CTLs Following Stimulation by DCs Pulsed with Early Explant Tumour Peptides 64

Figure 2-8 Cr^{51} Release assays for Cultured Tumour Cells 65

Figure 2-9 Cr^{51} Release assay for Autologous Fibroblasts 66

Figure 3-1 Trial Study Design 83

Figure 3-2 Trial Timeline for EPIC 001 84

Figure 3-3 Trial Timeline for EPIC 004 85

Figure 3-4 Trial Timeline for EPIC 006 86

Figure 3-5 Trial Timeline for EPIC 007 87

Figure 3-6 Patient Mixed Lymphocyte Proliferation 94

Figure 3-7 Interferon Gamma Secretion by Patient Mixed Lymphocytes 96

Figure 3-8 Mixed Peripheral Lymphocyte Cytotoxicity Against Autologous Tumour Cells 97

Figure 3-9 Trial Patient Serum ESR Levels Versus Time 99-100

Figure 3-10 Trial Patient C Reactive Protein Levels Versus Time 101-02

Figure 3-11 Trial Patient Lactate Dehydrogenase Levels Versus Time 103-04

Figure 3-12 Trial Patient Serum S100β Levels Versus Time 105-06

Figure 3-13 Immunohistochemistry EPIC 001 112

Figure 3-14 Immunohistochemistry EPIC 004 114

Figure 3-15 Immunohistochemistry EPIC 006 116

Figure 3-16 Immunohistochemistry EPIC 007 118-19

Figure 4-1 Schematics of M6PR 146

Figure 4-2 CI-M6PR Expression on Tumour Cell Lines 162

Figure 4-3 CD-M6PR Expression on Tumour Cell Lines 163

Figure 4-4 Confocal Images of CI-M6PR Localization 164

Figure 4-5 Evaluation of GrB-OG Uptake 166

Figure 4-6 TUNEL Assay of GrB Mediated Apoptosis of Tumour Cells 167

Figure 4-7 Western Blot of Apoptosis Related Proteins 168

Figure 4-8 Western Blot of Anti-apoptosis Proteins 169

Figure 4-9 CTL Expression of the CI-M6PR 171

Figure 4-10 DC Expression of the CI-M6PR 172

Figure 4-11 Confocal Images of CI-M6PR Localization in Immature DCs 173

Figure 4-12 Confocal Images of CI-M6PR Localization in Mature DCs 174

Figure 4-13 TUNEL Assay of GrB Mediated Apoptosis of CTLs and DCs 175

Thesis Abbreviations	
Abbreviation	**Full Text**
AA	anaplastic astrocytoma
AJCC	American Joint Committee on Cancer Staging
APC	antigen presenting cell
ATCC	American Type Culture Collection
BCG	Bacillus Calmette-Guerin
bFGF	basic fibroblast growth factor
CD	cluster of differentiation
CD-M6PR	cation dependent mannose 6 phosphate receptor
CI-M6PR	cation independent mannose 6 phosphate receptor
CNS	central nervous system
COX-2	cyclooxygenase-2 enzyme
CRP	C-reative protein
CT	computed tomography
CTL	cytotoxic T-lymphocyte
CTLA-4	cytotoxic T lymphocyte associated antigen 4
DC	dendritic cell
DMEM	Dulbecco's modified Eagle's media
DNA	deoxyribose nucleic acid
DTH	delayed type hypersensitivity
DTIC	dacarbazine
EDGF	epidermal derived growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunosorbent assay
ELISPOT	enzyme linked immunosorbent spot
ESR	erythrocyte sedimentation rate
FACS	flow automated cytometric stream
FADD	Fas associated death domain
FAMM	focal atypical multiple moles/melanoma moles
FBS	fetal bovine serum
FEBRT	focused external beam radiation therapy
GBM	Glioblastoma Multiforme
GFAP	glial fibrillary associated protein
GM-CSF	granulocyte monocyte-colony stimulating factor
GrB-OG	granzyme B labelled with Oregon green
HBSS	Hank's buffered saline solution
HIV	human immunodeficiency virus

Thesis Abbreviations	
Abbreviation	**Full Text**
HLA	human leukocyte antigen
HPLC	high pressure liquid chromatography
ICAM-1	intracellular adhesion molecule - 1
IFNγ	interferon gamma
IGF	insulin-like growth factor
IL	interleukin
KLH	keyhole lympet hemocyanin
LAK	lymphokine activated killer cell
LAMP	lysosomal associated membrane protein
LDH	lactate dehydrogenase
LFA-1	lymphocyte function-associated antigen-1
LOH	loss of heterozygosity
MAGE	melanoma antigen E
MHC	major histocompatability complex
MIB1	mindbomb homologue 1
MRI	magnetic resonance imaging
NK	natural killer cells
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered solution
PCNU	1-(2-chloroethyl)-3-(2,6-dioxo 3-piperidyl)-1 nitrosurea
PDGF	platelet derived growth factor
PGE2	prostaglandin E2
PTEN	phosphatase and tensin homologue, a tumour suppressor
PVC	procarbazine, vincristine, lomustine
RPMI	Rosewell Park Memorial Institute
SK 24 Mel	Sloan Kettering Melanoma 24
TAP	transporters associated with antigen processing
TCR	T-cell receptor
TGFβ	transforming growth factor beta
TIL	tumour infiltrating lymphocyte
TNFα	tumour necrosis factor alpha
TRAIL	TNF related apoptosis-inducing ligand
uPAR	urokinase-type plasminogen activator receptor
UV	ultra violet
VEGF	vascular endothelial growth factor
VP-16	etoposide

Thesis Abbreviations

Abbreviation	Full Text
WBRT	whole brain radiation therapy

CHAPTER 1

Treatment of Malignant Melanoma and High Grade Gliomas

Introduction:

Brain tumours account for approximately 2% of the cancer mortality in men and 1.4% of the mortality in women, and within the age group 15 to 34 years, they are the third most common cause of death due to cancer[1]. Intracranial cancers can be roughly divided as 2/3 primary neoplasms, and 1/3 metastatic lesions. By far the most common type of brain tumours are those of neuroepithelial origin. Gliomas which are tumours arising from astrocytes, oligodendrocytes, ependymal cells, and choroid plexus cells account for about 50% of primary brain neoplasms with an over all incidence between 5 to 10 per 100,000 in the general population[2]. The 1993 World Health Organization classification system grades these tumours from I – IV[3]. Grade IV is the most malignant and is also referred to as glioblastoma multiforme (GBM). Unfortunately this grade is also the most common grade at diagnosis. In the 45-65 year old group 71% of brain cancers were high grade gliomas whereas 21% were defined as low-grade[2]. It is well recognized that higher grades of gliomas are associated with poorer prognosis. On the one hand, grade I astrocytomas treated with subtotal resection have a 10 year survival rate of about 80%[4]. On the other hand, the two year survival rate for grade IV tumours is 3-11% [5,6] with a mean survival even with maximal therapy of less than one year[7,8]. Despite recent advances in diagnosis and treatment, the incidence of and mortality from glioma may be increasing in many developing countries,[9,10] especially among the elderly[2,11]. However, it is likely that part of the elevated incidence is due to increased surveillance and access to diagnostic imaging.

The neuropathologist H. Scherer first described two classes of glioblastoma based on their biological and clinical behaviour[12]. Kleihues and Ohgaki have provided an extensive summary of the genetic pathways that are associated with generation of primary or secondary glioblastoma phenotype[13]. Approximately 95% of glioblastomas are classified as primary or *de* novo, having no clinical or histopathologic evidence of prior low grade[14]. For secondary glioblastomas, that is those that have a more prolonged clinical course and evidence of evolution from lower grade, there have been key genetic alterations more prominently identified in association with this class of histological disease progression. Mutation of p53, for example, is found in up to 65% of low grade astrocytomas whereas it is seen in only 28% of primary GBMs[15,16]. Loss of heterozygosity (LOH) of chromosome 10, the location of several putative tumour suppressor genes, is the most common genetic alteration in both primary and secondary glioblastomas. Interestingly though the loss of heterozygosity profile is different for the two tumour classes. LOH of 10p is present most prominently in primary glioblastomas[17]. Mutation of phosphatase and tensin homologue gene (PTEN), another locus on chromosome 10, is also much more prevalent in primary GBMs[18]. LOH 10q25-qter on the other hand is more common in lower grade astrocytomas[19]. Other mutations involving LOH at 22q[20] and LOH 19q[21] are identified much more frequently in association with secondary glioblastomas. Genetic alterations resulting in epidermal growth factor receptor (EGFR) amplification[22] or over expression[23] is typical in primary but not secondary GBMs. More recently, proteomics studies have identified that likely unique genetic alterations also result in over production of vascular endothelial growth factor

(VEGF) predominantly in primary glioblastomas and higher levels of platelet derived growth factor (PDGF) in secondary glioblastomas[24].

Very little is known about the causes of gliomas apart from a few very rare genetic syndromes. Hereditary syndromes in which glioma is known to frequently occur can only explain <5% of glioma cases[25]. The best defined involve specific germline mutations, and include Li–Fraumeni syndrome, neurofibromatosis 1 and 2, tuberous sclerosis, von Hippel–Lindau and Turcot's syndrome. To date there are no established causes of glioma, nor have any major environmental or lifestyle factors been identified that could be amenable to public health or lifestyle interventions[1].

Another important tumour of neuroepithelial origin is melanoma. This is classified as a secondary brain tumour. However, melanoma has, in fact, the highest predilection for metastasis to brain with 40 to 68% of patients harbouring intracranial lesions[26,27]. Currently the American lifetime risk of being diagnosed with invasive melanoma is 1.91% in caucasian males and 1.37% in caucasian females which correlates with an incidence rate of about 1 in 75 individuals[28,29]. In the United States melanoma is the sixth most common cancer in men and the seventh in women[30]. In Australia it is the leading cause of cancer in 15 to 44 year olds[31]. Of particular concern is that worldwide there has been a 3 to 7% annual increase in melanoma incidence in many countries since the 1960's[32-34]. Melanomas may be classified by either the Breslow thickness scale, the Clark's level, or the American Joint Committee on Cancer Staging (AJCC) scale[35]. The AJCC stages range from '0' – tumour *in situ* with no positive nodes and no metastases, to 'IV' – tumour of any thickness but associated with positive nodes and presence of metastases[36]. A review of over 84,000 cases of melanoma in the U.S. between 1985 and 1994 revealed the AJCC stage incidence of melanoma to be 0 – 14.9%, I – 47.7%, II – 23.1%, III – 8.9%, and IV – 5.3%[37]. In the same study patients who presented with metastasis with an unknown primary (Stage IV) despite surgical excision had a 5 year survival of 38.8%. Patients with central nervous system (CNS), bone, liver, or multiple sites of first metastases have the poorest progrnosis[38]. When cerebral metastases have been identified the mean survival is 113 days[39]. The most widely accepted causative agent for melanomas is ultra violet (UV)/sunlight exposure[40]. It is also clear that individuals of fair complexion are more vulnerable to developing melanomas as evidenced by higher incidence in Australia, and New Zealand and in Northern Europe and among American Caucasians. Melanomas arise either de novo or by progression from nevi, especially dysplastic nevi. Approximately 30% of melanomas arise from a preexisting nevus[41]. The presence of nevi and a family history of melanoma are strongly associated with development of the disease[42]. In addition to these characteristics and environmental causes a few genetic syndromes have been associated with melanomas. These are Xeroderma Pigmentosum, Li-Fraumeni and FAMM (Focal Atypical Multiple Moles/Melanoma Moles), and Albinism[43].

Current surgical, radiation, and chemotherapy strategies:

Clinically both high gliomas and melanomas have a bleak prognosis even with aggressive treatment. For gliomas the purpose of surgery is to provide adequate tissue for accurate diagnosis and to reduce tumour burden. It has been demonstrated that the extent of tumour resection has some impact on survival, though in a limited way. In one study evaluating residual supratentorial GBMs or anaplaplastic astrocytomas tumour using post operative computed tomography (CT) scans it was found that median survival with gross total resection of was 90 weeks compared to only 43 weeks with subtotal resection[44]. In a similar study utilizing post operative magnetic resonance imaging (MRI) it was shown that the presence of residual tumour was associated with a 6.595 times higher risk of death compared to patients without residual tumour[45]. The value of re-operation for tumour recurrence is controversial among neurosurgeons. Here the guiding principle is reduction of tumour burden with a view to palliation of symptoms or increasing efficacy of adjunctive therapies such as radiation or chemotherapy. The median survival of GBM patients following a second tumour resection is 14 to 36 weeks[46]. The most definitive study to date regarding surgical intervention for malignant gliomas was provided by the Glioma Outcome Project[47]. In this multicentre prospective study involving 565 patients with grade III or IV gliomas, it was found that the overall median survival from diagnosis was 48.2 weeks. Of particular importance was the finding that median survival for those who underwent biopsy only was 27.1 weeks compared to 51.6 weeks for patients that underwent resection.

Due to the overall poor prognoses for high grade gliomas despite surgical intervention, additional treatment options including radiation and or chemotherapy are also employed but with relatively little impact on the ultimate life duration of patients[48]. Radiation therapy is the most common treatment performed on patients harbouring high grade gliomas with whole brain external beam radiation being the most frequently utilized modality by far[49]. Radiation therapy is limited by the significant side effects of radiation induced necrosis, inflammation, and damage to normal brain parenchyma. Significant efforts have been given to determining protocols that limit this toxicity. Radiation, however, does add significantly to patient life expectancy. In one study involving 222 patients, the median survival time with surgery alone was 14 weeks compared to 36 weeks when surgery and whole brain radiation (WBRT) were combined[50]. Among the very best results of combined treatment with radiation is a study involving 171 patients that revealed a median survival of 62 weeks for WBRT plus PCV (procarbazine, vincristine, lomustine) in contrast to 42 weeks for surgery followed by PCV[51]. Application of newer radiotherapy protocols has not significantly improved outcomes. In 346 patients that underwent focused external beam radiotherapy (FEBRT) in combination with PCNU [1-(2-chloroethyl)-3-(2,6-dioxo 3-piperidyl)-1 nitrosurea] the median time of survival was 47 weeks[52]. In a small study of 31 patients that after surgery underwent EBRT followed by a boost by Gamma knife-stereotactic radiotherapy it was shown that median survival was extended to 25 months compared to 13 months with EBRT alone[53]. It will remain to be seen if this result is repeatable in a larger population of selected patients. An extensive systematic review of radiation therapy protocols for malignant gliomas has determined ultimately that there is a significant survival benefit to external beam radiotherapy following surgical excision[54].

In general chemotherapy agents alone do not confer a survival benefit and are clinically always combined with radiation treatment. A meta-analysis involving 3004 patients demonstrated that there was only a 2 month increase in median survival time and an increase in 1 year survival from 3 to 9% when radiation treatment alone was compared with combined radiation and chemotherapy[55]. Newer drugs such as the alkylating agent temozolomide which has excellent penetration characteristics and a lower side effect profile has not demonstrated a substantial increase in efficacy with median survival rates of 5.4 months for recurrent GBM and 13.6 months for anaplastic astrocytomas (AA) respectively[56-58]. The addition of daily temozolamide chemotherapy to fractionated radiation (to a total of 60Gy) has demonstrated improving median survival in GBM patients from 12.1 months to 14.6 months in a multicentre prospective randomized trial involving 573 patients[59]. As a result temozolamide adjunctive therapy has become the treatment of choice for high grade gliomas. It has also been determined that a subgroup of malignant glioma patients, about 45%, express a methylated form of the O^6-methylguanine-DNA methyltransferase promoter which is associated with a substantial survival benefit[60]. Furthermore, it has also been suggested that protracted low dose temozolamide treatment may cause depletion of O^6-methylguanine-DNA methyltransferase and thereby enhance the activity of the agent[61]. Prolonged administration of temozolamide has however been associated with a risk of myelosuppression and in particular thrombocytopenia in up to 10% of patients[62,63]. Another newer agent etoposide (VP-16) targets the deoxyribose nucleic acid (DNA) repair enzyme topoisomerase II. Radiation followed by a course of etoposide resulted in a median survival time of 12 months[64]. When patients are treated with intra-arterial etoposide prior to radiation median survival is 20 months compared to 7 with concurrent treatment[65].

The primary treatment for melanoma is surgery. The grade at the time of surgical excision is strongly correlated with survival. For melanoma *in situ* (grade IA) 5 year survival is 93% whereas for stage IV it is less than 10%[66] The presence of disease in sentinel nodes is possibly the most important survival predictor. When nodes are negative 5 year survival rates are about 80% compared to less than 40 % when a positive sentinel node is identified[67,68]. Despite negative examination for nodal involvement stage IIB melanoma is associated with 60 – 70 % incidence of metastases to the regional lymph nodes and 30 – 50 % incidence of distant metastases[69]. In a recent series of 236 patients with stage III melanoma, the addition of therapeutic lymph node dissection in addition to primary tumour excision did not improve survival with the 5 year overall survival rate observed to be 26%[70]. For patients with identified intracranial melanoma metastases the median survival is 113.2 days despite surgical excision and radiation[39]. Craniotomy and excision of a single CNS metastasis with absence of other visceral metastasis was associated with longer survival.

Radiation therapy for melanomas has limited efficacy and in general is used for palliation and reduction of local recurrence following surgery[71]. In a comparison of surgery versus surgery followed by WBRT, the outcomes for the treatment groups were not significantly different (p=0.67) with median survival being only 6.7 months[72]. WBRT though not improving survival significantly does decrease the incidence of CNS recurrence from 69% to 37%[73]. The application of newer techniques of stereotactic radiosurgery for intracranial melanoma has not significantly improved survival[74,75]. In a retrospective study of 74 patients the addition of stereotactic radiosurgery following WBRT extended median survival from 4.8 to 8.8 months[76].

The activity of chemotherapeutic drugs against melanomas is limited. Dacarbazine (DTIC) an alkylating agent is the most active single agent for treatment of metastases. However, it is associated with a response rate of 20% and duration of response of only 6 months[77-79]. Combination therapy involving dacarbazine, carmustine, cisplatin, and tamoxifen in two studies involving 20 patients demonstrated a 50% response rate and 15-20% cure rate[80,81]. In a prospective phase II study of 79 patients with metastatic melanoma application of this regimen resulted in a response rate of 15%, with mean response duration of 8 months, and mean survival time of 9 months[82].

Introduction to Immunotherapy for Malignant Melanoma and High Grade Gliomas:

Because of the poor prognosis for patients with these tumours of neuroepithelial origin, despite maximal intervention with currently available surgical techniques, and chemotherapeutic and radiation protocols, newer modalities of treatment are actively being explored. One such paradigm is immunotherapy which seeks to manipulate the immune system to recognize hereto unrecognized tumour cells and generate a specific cytotoxic response.

The immune system, in brief, utilizes an innate and acquired system of preventing systemic access by pathogens or abnormal cells. The innate system comprised primarily of natural killer (NK) cells, immunoglobulins, and complement primarily provides a rapid response to foreign particles and cells that do not present self major histocompatability complex (MHC), leading to rapid opsonization and removal. The acquired immune system is comprised of a carefully orchestrated interaction between cells specialized to recognize, sequester, and present foreign or abnormal antigens (antigen presenting cells (APCs) such as macrophages or dendritic cells (DCs), effector cells such as CD 8+ (cluster of differentiation 8) cytotoxic T-lymphocytes, CD 4+ helper T-lymphocytes and CD 25+ T-regulatory lymphocytes. Though the innate immune system provides a first line, relatively non-specific response, the acquired immune system is highly specific, adaptable to new and varied pathogens, and has the important quality of generating memory in order to provide specific and rapid response to subsequent antigen exposure.

In order to generate a specific acquired cellular immune response it is now recognized that three events must occur. First, antigen must be presented of the surface of an antigen presenting cell in the context of MHC for recognition by an antigen specific T-lymphocyte. Second, there must be binding of co-stimulatory molecules present on the surfaces of the APC and the T-lymphocyte. Third, success of the first two steps results in signal transduction activation that ultimately leads to interleukin 2 (IL-2) expression and secretion. The presence of IL-2 is a requirement for the activation and proliferation of clones of effector and memory lymphocytes.

Melanomas and gliomas like most tumours have evolved multiple strategies to avoid immune detection and to truncate immune responses. Both tumour types have been observed to have reduced ability to present antigen in the presence of MHC [83-85]. Another common and shared strategy of these tumours is the secretion or expression of molecules that not only promote tumour growth but also dramatically impair immune response by T-lymphocytes and APCs[86-88]. Both melanomas and gliomas express high levels of transforming growth factor beta (TGFβ) which is associated with immune cell suppression and decreased MHC class II presentation[89-93]. Likewise prostaglandin E_2 (PGE_2) impairs T-cell responses and inhibits the production of immune promoting cytokines[94,95]. IL-6 is also expressed by these tumours which interferes with interferon gamma (IFNγ) mediated Th1 responses[96,97]. Both tumours also secrete IL-10 which is a powerful inhibitor of APC stimulation and is recognized to contribute to immune tolerance[98-102]. Together these examples clearly underscore the complex environment in which tumours succeed and avoid eradication by the host immune system.

Immunotherapy, in general, then strives to overcome some of these obstacles in order to tip the balance in favour of the host immune system's ability to detect and respond to tumours with specific cytotoxic activity. For the treatment of cancer, immunotherapy strategies have fallen into two broad categories. Non-Specific therapy -

which utilizes cytokine/adjuvant therapy, and Specific therapies - which include antitumour antibodies, adoptive, and active immunotherapy.

The first of these involves the use of immunoadjuvants to directly affect tumour cell phenotype, or to indirectly modify a patient's systemic immunosuppressive profile. One of the limitations of immunoadjuvant is the side effects of the various agents used. However, many such agents are used with clinical safety and efficacy. IL-2 as an adjuvant for melanoma treatment is well established and is part of many well established therapeutic protocols. IL-2 acts primarily as a T-cell growth factor but is also able to reverse IL-10, superantigen, and CD4+CD25+ T-regulatory cell induced effector T-cell anergy[103-105]. IL-2 treatment for gliomas has mainly been employed in conjunction with lymphokine activated killer cell (LAK) protocols [106,107]. Interferon is used as an immunoadjuvant because of its ability to activate innate immune cells, reduce tumour cell PGE_2 secretion, antagonize malignant cell TGFβ function, and enhance the apoptotic potential of tumour cells[108-113]. In glioma patients the use of IFN alone has demonstrated limited clinical efficacy[114,115]. It may find a more important role in combination with other therapies such as chemotherapy[116]. Granulocyte monocyte – colony stimulating factor (GM-CSF) as an immunoadjuvant has been observed to promote recruitment of CD34+ progenitor DCs, and stimulation of DC proliferation, maturation, and expression of : CD40, tumour necrosis factor alpha (TNFα), MHC class II, and CD86 co-stimulatory molecules, all of which are pivotal in generating effector and memory T-lymphocyte responses[117-122]. The recognition of the importance of cyclo-oxygenase (COX) receptor expression in tumour cells and its putative role in tumourgenesis has led to the use of COX inhibitors in adjuvant therapy. Specifically, COX-2 inhibitors are utilized because of their recognized ability to reduce malignant cell proliferation and invasion, tumour cell secretion of PGE_2 (a potent APC and T-cell suppressant) and antagonize both vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) induced angiogenesis[123-126]. Retinoic acid has been used in the treatment of melanoma due to its promotion of tumour cell differentiation, observed influence in decreasing tumour cell proliferation and metastatic potential, down regulation of matrix metalloproteinases (MMPs), decreasing the levels of insulin-like growth factor binding protein 2, and association with increasing apoptotic sensitivity of melanoma cells[127-130].

The second broad category of anti-cancer immunotherapy involves specific antitumour targeting. One such approach is passive serologic treatment which utilizes monoclonal antibodies to target epitopes/antigens specific to tumour cells and expressed in abundance on those cells. Monoclonal antibodies can bring about tumouricidal effects either by opsonizing tumour cells and subsequently activating other components of the immune system such as complement cascade and phagocytes, or by serving as a delivery vehicle for various toxins. To date antibody based treatments have not demonstrated significant efficacy in clinical application but is part of ongoing investigation.

The key element required in antibody mediated strategies is the identification of antigen that is specific to the target tumour cell. Mutated epidermal growth factor receptor (EGFR), in particular EGFRvIII is such an example that is expressed in a high percentage of malignant gliomas and is associated with tumourigenicity[131,132]. In recent animal model studies antitumour efficacy has been suggested against B16 melanomas transfected to express EGFRvIII in mice[133], and against rat gliomas transfected to express human EGFRvIII and targeted by antibody linked with boronated cetuximab[134]. A phase 2/3 clinical trial of Celldex, CDX-110, human monoclonal antibody against EGFRvIII is currently recruiting patients in a multicenter trial.

Tenascin is another example of a relatively specific tumour target. It is an extracellular matrix protein whose deposition is reported in up to 90% of gliomas[135]. Using radiolabelled iodine^{131} murine monoclonal antibody administration into the resection cavity of newly diagnosed malignant gliomas resulted in median survival times of 69 to 79.4 weeks for patients with GBMs in human phase I and phase II trials respectively[136,137]. Some important considerations about this treatment method despite these encouraging results are the 27% incidence of reversible hematologic toxicity and 15% occurrence of treatment related neurologic toxicity. In addition other investigators have reported up to 69% of patients develop human antimouse antigens[138].

Recent increasing understanding of the role of regulatory T lymphocytes in mediating normal self-tolerance and immunosuppression in tumours has generated interest in specific targeting strategies against these cells. An example of this is targeting of the immunomodulating molecule cytotoxic T lymphocyte associated antigen 4 (CTLA-4)[139]. Maintenance of immunosuppressive influence by regulatory T lymphocytes is dependant upon CTLA-4 expression[140,141]. In a murine glioma model Sampson et. al. employed a monoclonal antibody against CTLA-4 and found increased survival in treated mice[142]. In clinical trials of CTLA-4 antibodies in patients harboring grade IV melanomas a decrease in numbers of circulating regulatory T lymphocytes was reported. However, the significant incidence of grade III and IV autoimmune toxicity and no objective clinical anti-tumour response, suggests this strategy is not yet ready for wider clinical application[143,144].

Another specific type of immunotherapy is adoptive immunotherapy. This strategy involves the isolation of autologous immune cells which are then stimulated *ex vivo* before being injected back into the patient. In the simplest form of this treatment, circulating lymphocytes are activated and expanded *ex vivo* with IL-2. These LAKs have however been ineffective in the treatment of gliomas and melanomas and this has been abandoned as a treatment modality[145-148].

An additional approach has sought to take advantage of lymphocytes that are infiltrating surgical tumour resections with the presumption that they represent T-cells specific for antigen targets within the tumour. These tumour infiltrating lymphocytes (TILs) can be grown from a tumour cell suspension in IL-2 containing media[149,150]. The largest applications of this strategy for metastatic melanoma involved the treatment of 86 and 134 patients with TILs and high dose IL-2[151,152]. It was found that about one third of patients had only partial and short lived responses. Poor clinical outcomes with this treatment continue despite the objective finding that radiolabelled IL-2 boosted

TILs localized to tumour sites *in vivo*[153]. For the treatment of gliomas the use of TILs had not extended beyond largely unsuccessful animal studies[154]. However, a recent modification of this approach combining vaccination and harvest of lymphocytes for surgically excised draining lymph nodes may hold some promise[155]. In this study patients were injected with autologous irradiated tumour cells. Lymphocytes harvested from excised draining lymph nodes were then expanded *ex vivo* before re-infusion. Some positive responders were noted in this phase I trial.

The most promising adoptive immunotherapy technique involves the isolation, activation, and re-injection of autologous dendritic cells (DCs). DCs function as dedicated antigen presenting cells (APCs) and are recognized as the most powerful regulators of the immune system[156]. They possess the unique ability to activate and modulate both the innate immune system, in particular NK and NKT-cells and the acquired immune system including CD 8+ cytotoxic T-lymphocytes and CD 4+ helper T-lymphocytes. DCs originate from the bone marrow but migrate to nearly all organ systems to function as immune sentinels. They represent approximately 0.3% of the circulating lymphocyte population. The immature DC functional phenotype is to acquire antigen and process it for presentation in the context of MHC. The mature DC phenotype is characterized by down regulation of antigen processing machinery and development of immune system stimulatory capacity, typified by markedly increased expression of MHC, as well as presentation of co-stimulatory markers (CD 80, CD 83, CD 40, DC-LAMP), adhesion molecules (CD 54, CD 58) and release of cytokines/chemokines (IL-2, IL-12, IL-15)[157-162].

The clear attributes of DCs as powerful immune modulators has made them a natural focus of cancer immunotherapy strategies. Since the discovery and standardization of techniques to generate DCs from circulating monocytes, the application of autologous DC preparations in clinical trials for melanomas and gliomas has blossomed. The focus of these trials has been both safety and the resolution of the key question of what form of antigen presented to DCs will ultimately generate the most specific and clinically relevant anti-tumour response. The array of options for antigen include: tumour specific peptides such as melanoma antigen E-1 (MAGE 1), tumour lysates, acid eluted peptides, naturally processed peptides, apoptotic bodies, DC-tumour fusion bodies, total tumour RNA or DNA, and tumour exosomes.

The majority of clinical DC trials for melanoma patients have utilized autologous DCs primed with melanoma specific antigens[163-165]. The main criticisms of this approach are the limitation of the range of epitopes provided to DCs, by antigen or patient human leukocyte antigen (HLA) type restriction, and failure of some malignant cells within the target tumour to express the selected antigen. As an example, Schuler-Thurner et. al. [164], using MAGE 3 peptide, were able to observe induction of IFNγ producing CD8+ T-cells *in vitro*, however they were unable to demonstrate either a significant clinical response, or MAGE 3 specific cytotoxic T-lymphocyte (CTL) enzyme linked immunosorbent spot assay (ELISPOT) reactivity in fresh patient blood samples.

Two important studies have looked at the use of autologous melanoma tumour lysate to prime DCs for antigen presentation. The landmark study by Nestle et.al. looked at patients vaccinated with peptide versus tumour lysate pulsed DCs and found that 2 of 4 "lysate primed" patients had a clinical response and 4 of 4 demonstrated delayed type hypersensitivity (DTH) reaction to the adjuvant keyhole limpet hemocyanin (KLH)[166]. Recently, Hersey et. al. compared 19 patients treated with autologous DCs and tumour lysates with 14 patients treated with DCs pulsed with melanoma specific peptides[167]. These authors report 4 of 19 patients in the lysate treated cohort showed partial response compared to no responses in the group treated with melanoma specific peptide. These findings suggest that the use of autologous tumour lysate has an apparent clinical advantage over treatment with specific peptide pulsed DCs.

All clinical trials utilizing DC based vaccinations for Gliomas have utilized strategies which have sought to provide a more complete antigen array for stimulation of cytotoxic anti-tumour responses. Yu et. al. performed the first clinical trial of a DC based vaccine for the treatment of high grade gliomas[168]. In this trial patients underwent a full course of external beam radiation therapy (EBRT) after tumour resection before being immunized with autologous peripheral blood derived DCs pulsed with acid eluted MHC class I associated peptides obtained from cultured autologous tumour cells. The authors reported a mean survival in the vaccinated cohort of 455 days compared to 257 days for patients undergoing surgery and radiation treatment alone. Interestingly, they observed for the first time, that tumour samples from subsequent resections in patients with recurrences and who had been vaccinated had infiltration of both CTLs and CD45RO+ memory cells. Neither of these cell types had been present in tumour sample harvested before treatment.

Liau et. al. employed a similar strategy involving DCs pulsed with acid eluted tumour peptides and found that despite 6 of 12 patients having measurable systemic CTL responses by enzyme linked immunosorbent spot (ELISPOT) and *in vitro* cytolysis assays, there were no objective clinical responses or increased survival[169]. The authors point out that diminished immune responses were correlated with tumours expressing high levels of TGF-β2.

In order to address the concerns that acid-eluted peptides are MHC class I restricted and as such provide tumours with downregulated MHC I expression a means of escape from vaccine induced CTL responses, several authors have used tumour cell lysates to pulse DCs. This strategy strives to diversify immune responses via both MHC class I and class II antigen presentation[170-175]. For example, Rutkowski et.al. using autologous tumour homogenate, treated 12 patients with recurrent malignant gliomas following surgical resection[175]. Remarkably, the authors report tumour response to immunotherapy in 4 of 12 patients with 2 patients having complete remission sustained at 3 years. Wheeler et. al. also demonstrated that a tumour lysate-DC vaccination could be safely combined with conventional chemotherapy[170]. In fact the authors observed that treatment with DC vaccination prior to chemotherapy resulted in longer overall survival.

Akasaki et. al. reported strong CTL and NK cell infiltration of glial tumours in a murine glioma model treated with apoptotic tumour cell-DC fusion vaccination[176]. On the basis of this encouraging report Kikuchi et. al. has applied this technique in clinical trials. In their first trial the authors report similar findings of elevated populations of CD 56+ NK cells amongst circulating peripheral blood monocytes[177]. In a second trial the authors compared the addition of IL-12 adjuvant therapy to treatment with tumour-DC fusion vaccination alone, and though not observing in either treatment group the elevation of circulating T-, B-, and NK- cell populations, patients that had been treated with IL-12 did show some clinical advantage[178].

Despite the fact that DC based vaccination treatments for cancer patients are well tolerated, to date limited clinical responses necessitate ongoing research to refine the best therapeutic regimen to bring about both strong antigen specific anti-tumour responses and improved clinical efficacy. In this thesis we present our work in developing an autologous dendritic cell vaccine consisting of autologous peripheral monocyte derived dendritic cells pulsed with naturally processed peptides from autologous early explant cultured tumour cells for the treatment of malignant melanomas and high grade gliomas. In Chapter 2 we provide our *in vitro* data on the characterization of the vaccine components and efficacy of autologous of HLA-A2 matched dendritic cells to induce cytotoxic T-lymphocyte responses against melanoma and glioma tumour cells. In Chapter 3 we provide an interim progress report on our Phase I, Health Canada approved trial of an autologous dendritic cell vaccine for patients with medically refractory malignant melanoma in combination with a unique and comprehensive immunoadjuvant protocol. We have also been interested in investigating ways in which both melanomas and gliomas avoid apoptosis. In particular, the role of the cation independent mannose 6 phosphate receptor (CI-M6PR), which is recognized to mediate in part granzyme B initiated apoptosis, has not previously been investigated. In Chapter 4 we report our evaluation of the role the CI-M6PR plays in avoidance of cytotoxic T-cell induced apoptosis by glioma and melanoma cell lines.

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Chapter 2

Human Dendritic Cells Primed with Glioma or Malignant Melanoma Naturally Processed Tumour Peptides Induce Cytotoxic Antitumour Response *In Vitro*

Introduction:

Malignant astrocytomas and metastatic melanomas are devastating neoplastic diseases characterized by exceedingly rare cure rates, short survival times and significant tumour progression-related morbidities. Despite advances in surgical technique, chemotherapeutics, radiation therapy, and aggressive multimodality therapies, they continue to have a dismal prognosis. Melanomas share with gliomas: a common embryologic origin - the neuroectoderm, a number of identified characteristic tumour markers such as gp100, melanoma antigen E-1 and E-3 (MAGE-1&3), abnormal or inhibited tumour suppressor genes such as p53 and phosphatase and tensin homologue (PTEN), and activation of specific oncogenes such as Ras, Tert, platelet derived growth factor (PDGF), epidermal derived growth factor (EDGF)[1].

These tumours share with all malignancies an ability to avoid immune surveillance[2]. Briefly – cancer cells present a profile of low immunogenicity by exhibiting very low, or no major histocompatability complex (MHC) class I molecules and/or specific epitopes on their surfaces which are necessary for them to be recognized as foreign and elicit an immune response. Furthermore, they lack surface expression of co-stimulatory molecules required for induction of an immune response. Tumour cells also can generate a number of immune suppressing cytokines or exhibit a cytokine profile which promotes a humoural (Th2) rather than a cytotoxic (Th1) response. An antibody mediated immune response is very ineffective in eradicating solid tumours. Finally, tumour cells produce survival and anti-apoptotic proteins (eg. BCL2, XIAP, Survivin, etc.) that abrogate immune mediated killing.

Dendritic cells (DCs) are powerful immune regulating cells which play a pivotal role in linking innate and acquired immunity. They are professional antigen presenting cells that initiate potent T-cell responses against non-self antigens such as tumour associated peptides[3].

Immature DCs which specialize in antigen capture and processing can be generated from peripheral blood CD14+ monocytes of cancer patients by incubation of these cells with granulocyte macrophage – colony stimulating factor (GM-CSF) and interleukin-4 (IL-4)[4, 5]. Upon engulfment of antigen, DCs undergo a maturation process that involves foreign peptide (antigen) being packaged via TAP (transporters associated with antigen processing) and displayed in the context of MHC on the DC surface[6]. In addition, mature DCs strongly express co-stimulatory molecules required to initiate specific T-cell differentiation and clonal expansion of effector and memory cells[7-9], are resistant to the suppressive effects of IL-10, and synthesize IL-12 that enhances both innate and acquired immune responses[10].

Since the discovery of mature DCs' ability to cross-prime cytotoxic T-lymphocytes (CTLs) with exogenous antigens on MHC class I molecules[11, 12] considerable energy has been invested in defining effective methods of exposing potentially immunogenic antigens to DCs. A variety of methods have been utilized to provide tumour-associated antigens to DCs, including tumour lysates, apoptotic tumour cells, and pure tumour peptides[13]. The use of complex antigen in the form of large peptides derived from tumour cell freeze-thaw lysates, apoptotic tumour bodies, or tumour cell-DC fusions, pose the difficulty that antigen must undergo substantial processing once internalized by the DC before peptide can be presented by MHC molecules at the cell surface. Furthermore, such processing may bias presentation toward MHC class II and a Th2 (humoural) type immune response. The criticism of using selected specific antigens such as melanoma antigen E-1 (MAGE 1) or tyrosinase is that the resulting cross-priming of CTLs neglects subclones of cells within a tumour that do not express that particular antigen. The use of tumour surface antigens extracted by acid elution addresses this problem only in part, because the profile of antigens likely are those to which host immune tolerance and anergy have already been established. Application of such a technique did not yield a significant objective clinical response in a recent clinical trial[14].

To address the above shortcomings, we have utilized a unique method to extract a full spectrum of "naturally-processed" peptides which include cytosolic heat shock peptides from glioma and melanoma cells[15]. This method demonstrated activation of cytotoxic T-lymphocyte antitumour response against both autologous and allogeneic melanomas. The authors also demonstrated that the CTLs were specific for antigens other than the thirteen most commonly expressed melanoma specific antigens. In this study we generated extracts of naturally processed peptides from early explant cultures of surgically resected malignant gliomas and melanomas as well as established melanoma cell lines. We used these potentially highly immunogenic peptides to prime autologous or human leukocyte antigen A2 (HLA-A2) matched DCs. The objectives of this study were to determine if autologous or HLA-A2 matched dendritic cells primed with an array of naturally processed tumour peptides from malignant gliomas or melanomas could induce cytotoxic T-lymphocytes to kill *in vitro* target tumour cells from which the tumour peptides originated. Interested in the ability of this strategy to induce a strong and specific cytotoxic antitumour response against a wide range of potentially antigenic peptides we investigated the possibility of establishing this method for clinical application.

Methods:

Human Glioma and Melanoma Culture and Cell Lines

Early explant cultures were generated from glioma and melanoma surgical excision specimens. For Melanoma cultures, fresh operative tumour tissue was rinsed in Hank's buffered saline solution (HBSS) and minced into 2 to 3mm^3 pieces. The tissue was then incubated in an enzyme mixture consisting of 2 mg/ml collagenase II (Invitrogen) and 0.1 mg/ml hyaluronidase(Sigma) in HBSS for 1 hour at room temperature with continuous stirring. The resulting tumour slurry was passed through a cell strainer (100μm BD/Falcon). The tumour cells were layered on the density gradient media Ficoll-Paque Plus (Amersham) and centrifuged for 25 minutes at 640x g and 4°C. The cells in the interface layer were recovered and washed three times with HBSS. The tumour cells were then plated in T25 culture flasks with complete RPMI (Rosewell Park Memorial Institute) 1640 culture media supplemented with 2mM L-glutamine (Invitrogen) and 100μM sodium pyruvate (Invitrogen) and 10% fetal bovine serum (FBS) (Invitrogen). The cells were incubated humidified at 37°C and 5% CO_2.

For glioma explant cultures tumour tissue pieces were similarly[16] cut to 2-3mm^3 were incubated in an enzyme mixture consisting of 0.4 mg/ml DNase (Sigma), 0.5 mg/ml Pronase (Roche), and 0.25 mg/ml Collagenase IV (Invitrogen) at 37°C for 30 minutes followed by 4°C for an additional 30 minutes with gentle stirring throughout. The tumour cell slurry was then passed through a cell strainer. The cell suspension was then layered on the density gradient media Ficoll-Paque Plus and centrifuged at 400x g for 30 minutes at 4°C. The recovered cells of the interface layer were then washed three times in HBSS. Tumour cells were then plated in T25 culture flasks with Dulbecco's Modified Eagle's Media/ F 12 (DMEM/F12) supplemented with 10% fetal bovine serum, 100μM sodium pyruvate, 0.05 mM non-essential amino acids (Invitrogen), 2mM L-glutamine (Invitrogen).

The melanoma tumour line Sloan Kettering Melanoma 24 (SK 24 Mel) was obtained from the American Type Culture Collection (ATCC) and maintained in the RPMI media as described above.

Validation of Tumour Diagnosis

For each tumour sample definitive diagnosis was supported by the official clinical pathology report provided by the University of Alberta Hospital department of pathology. Melanoma cultures were further evaluated using the following antibodies; mouse anti-human fibroblast (Dako), mouse anti-human Melan A (Novo Castra NCL), and mouse anti-human malignant melanoma HMB45 (Biogenix). Glioma cultures were screened using the antibodies; bovine anti-rabbit glial fibrillary associated protein (GFAP) (Dako), and Mouse anti-human fibroblast (Dako). All antibodies were used in accordance with manufacturers' suggested method and visualized using horseradish peroxidase.

Generating Naturally Processed Tumour Peptide

We employed the method of isolating naturally processed tumour peptides as previously described by our collaborators[17]. In brief, the naturally processed tumour peptide extracts were generated from $1x10^8$ tumour culture cells. Cells were first rinsed several times in HBSS to remove any exogenous proteins contained in the media containing FBS. Next, 4 cycles of freeze thaw fragmentation in the presence of an acid extract solution consisting of 2mM TRIS/HCl, 10mM NaCl, and 0.1% trifluoroacetic acid were preformed. (It should be noted that this freeze thaw acid extraction of tumour peptide is quite different from the acid elution technique used by others, in which sequential pH change is used to isolate peptides from binding sites on surface MHC molecules on tumour cells[18, 19]). The cell lysate solution was then centrifuged at 1,250x g for 10 minutes at 4°C. The pooled supernatant was then centrifuged at 12,500x g for 15 minutes at 4°C. The supernatant once again was recovered and ultracentrifugation at 100,000x g for 50 minutes at 4°C completed. The supernatant was then placed in a 100 Dalton MWCO dialysis tube (Spectra/Por) which was submerged in a 1L PBS bath for 2 hours at 21°C. After 2 hours the dialysis bath was refreshed with 1L of PBS and dialysis was continued overnight (minimum 16 hours) at 4°C with continuous bath stirring. The dialysate was then recovered and loaded into a 5,000 NWML Ultrafree®-Cl filter (Amicon Millipore) and centrifuged at 1,900x g at 4°C for 4 hours. The resulting peptide extract was then filter sterilized with a µStar® LB 0.22 µm filter (Costar).

30µL peptide samples were submitted to *The Alberta Peptide Institute* for evaluation. In brief, peptides were analyzed by reverse phase chromatography using a Hewlett Packard 1090 chromatograph system. Samples were run on a Zorbax 300SB-C8 column (2.1 mm x 15 cm., Rockland Technologies) and monitored at 210 nm, using 0.1% trifluoroacetic acid/water (solvent A) and 0.1% trifluoroacetic acid/acetonitrile (solvent B). A gradient of 2% solvent B/minute at a flow rate of 0.2 ml/minute was used. 10µL samples of resulting peptide stock solutions underwent amino acid analysis. 10µL samples were freeze dried and dissolved in 6N hydrochloric acid containing 0.1% Phenol. Samples were hydrolyzed at 160°C for one hour. The hydrolyzed samples were dried under vacuum and dissolved in a Na-S buffer (Beckman). Aliquots were analyzed on a Beckman System 6300 with System Gold 8.1 data analysis. The peptide concentration of stock extracts was then calculated on the basis of amino acid content. Aliquots of peptide extract were frozen and stored at -80°C until required for priming of dendritic cells. We also independently evaluated the resultant peptide extracts for protein size by using a TRIS/Tricine 10-20% gradient gel.

Generating dendritic cells from peripheral blood monocytes)

Dendritic cells (DCs) are generated from peripheral blood mononuclear cells (PBMCs) isolated from peripheral blood samples from autologous (to the tumour) or HLA-A2 matched donors after the method previously described by our collaborators[17]. Whole blood was diluted 1:1 with HBSS and the resultant mixture layered over Ficoll-Paque®Plus (20ml diluted blood over 15 mL of gradient media) and then centrifuged at 400x g for 30 minutes at 18°C. The gradient interface was harvested and the isolated PBMCs washed 3 times in HBSS. The cells were counted and suspended in complete RPMI 1640, supplemented with 4 mM L-glutamine and 2% heat inactivated human AB+ serum (Bio-Whittaker) at a concentration of 7 x 10^6 cells/ml. The cells were incubated at 5% CO_2 and 37°C. After 2 hours all non-adherent cells were removed. The adherent monocytes were cultured in complete RPMI 1640 media supplemented with 1,000 IU/ml Granulocyte monocyte colony stimulating factor (GM-CSF from Immunex), 500 IU/ml IL4 (CellGenix), and 5% human AB+ serum. We also evaluated the generation of immature DCs under the influence of GM-CSF and interferon 2b (IFN2b). Cytokines were refreshed every 48 hours. As the PBMCs acquire DC characteristics they become non-adherent. On day 6 in culture the non-adherent cells were harvested, counted and plated at a density of 5 x 10^5 cells/ well in 2 ml of complete RPMI 1640 media in 6 well plates.

We evaluated two methods of initiating DC maturation of immature DCs generated by the above method. **1.** Immature DCs were cultured with 100μg/mL of tumour peptide extract for a period of 4 hours in standard culture conditions. After 4 hours inflammatory cytokines are added: 20 ng/ml IL-1β (CellGenix), 500 IU/ml IL-6 (CellGenix), 20 ng/ml tumour necrosis factor alpha (TNF-α from CellGenix), and 2μg/ml prostaglandin E_2 (PGE_2 from Amersham Pharmacia Europe). **2.** CD40 and LPS as stimulation agents were similarly utilized. The DCs were cultured with the maturing cytokines for 24 hours. The mature DCs were then harvested, washed once in HBSS, centrifuged at 200x g for 5 minutes at 4°C and then used for various experiments.

The immature and mature DCs were evaluated for morphology by light microscopy and for characteristic surface marker expression using both flow automated cytometric stream (FACS) analysis and confocal microscopy.

Flow Cytometry Evaluation of Dendritic Cells

DCs either mature or immature were harvested from there culture media in 6 well Corning culture dishes by gentle pipetting. The cells were pelleted (5,000 rpm x 5 minutes, 4°C), washed once in phosphate buffered saline (PBS) then suspended in immunofluorescence buffer (2% fetal calf serum, 0.02% sodium azide in PBS), and placed on ice.

Aliquots of 200μL were placed in 1.5 mL Eppendorf tubes and incubated on ice, in the dark, for one hour with the following antibodies (individually): 1μL mouse anti-CD80 IgG1 (Pharmingen), 1μL mouse anti-CD83 IgG1 (Pharmingen), 1μL mouse anti-CD14 IgG1 (ATCC), 1μL mouse anti- class II MHC labeled with FITC (Pharmingen), 1μL mouse anti-CD86 IgG1 RPE labeled (Caltag). For CD80, CD83, and CD14 detection after rinse with immunofluorescence buffer, cells were incubated with polyclonal goat anti-mouse IgG Cy3 labelled (Jackson Laboratories). After incubation, the cells were pelleted (2000 rpm x 5 minutes) and washed three times in immunofluorescence buffer. Finally cells were resuspended in 0.5 mL of 1% formalin in PBS. After staining, samples were read on a Becton-Dickinson flow cytometer and evaluated using either Lysis II or CellQuest 2.0 software.

Isolation of CD8 Lymphocytes

Autologous or HLA-A2 matched CD8+ lymphocytes (CTLs) were isolated using a Miltenyi magnetic/antibody coated microbead system – by positive selection (Miltenyi Biotech GmBH, Glsadback, Germany). Whole blood samples from HLA-A2 matched donors or patients were collected in heparinized vacutubes using standard phlebotomy technique. Approximately 20 mL of whole blood was diluted 50:50 with RPMI media and then layered over an equal volume of Ficoll-Paque in Falcon centrifuge tubes. The gradients were then centrifuged at 9,500x gravity for 10 minutes. The PBMCs at the gradient interface were then carefully harvested and washed three times with RPMI media. Approximately 1 x 10^7 cells were suspended in 80μL of sterile doing a viability count using Trypan blue activated clonally expanding CTLs were admixed at a ratio of 10: 1 with peptide primed DCs. Lymphocytes were cultured in RPMI 1640 supplemented with 10% heat inactivated pooled human serum, 2 mM L-glutamine and IL-2 at a final concentration of 20 IU/ mL. IL-2 was replenished at 48 hour intervals with a 50% media change. The CD 8+ve lymphocytes could be further expanded by additional rounds of antigen stimulation by mature peptide-primed DCs at weekly intervals.

ELISA for IFNγ

The supernatant from co-cultures of CD8 positive lymphocytes and mature peptide primed DCs was collected and analyzed by an enzyme linked immunosorbent assay (ELISA) for IFNγ concentration, an indicator of CTL activation. Production of IFN-γ was determined using an ELISA Quantikine kit (R & D Systems, Minneapolis, MN) according to the manufacturer's instruction. In brief, ($1\text{x } 10^5$) CD8 lymphocytes and peptide-pulsed autologous mDC ($1\text{x}10^4$) were incubated at 37°C in 96-well plate. A 50 uL sample of supernatant was harvested on day 2 and on day 6 of co-culture and assayed for IFNγ production. The amount of IFN-γ production was calculated based on the standard curve in the assay.

Chromium^{51} release assay

We evaluated HLA-A2 matched DCs and CTLs activated against SK 24 Mel (an ATCC well characterized melanoma line), and autologous DCs and CTLs activated against Ed 276Bt, Ed 326Bt, and Ed 343Mel (early explant tumour cultures: Ed =Edmonton, Bt = glioma, Mel = melanoma). Finally, efficacy of DC cross-priming of CTLs is determined using a standard Cr^{51} CTL assay.

In brief, autologous cultured tumor cells used as target cells were incubated with 100uCi Na_2 [^{51}Cr] O_4 (GE Amersham, Buck, United Kindom) for 45 min at 37°C. After extensive wash with HBSS, the chromium labeled tumour cells (target) were admixed with CD8 positive cells (effector) that had been activated by co-culture with peptide pulsed mDCs at an effector:target cell ratio of 20:1; 10:1; 5:1, and 2.5:1 in RPMI complete medium in triplicate wells of a round-bottom 96-well plate. After a 4 hour incubation, 100uL of the supernatant were collected and subjected to measure its radioactivity content. The specific percent of cytotoxicity was calculated based on the following formula:

% specific release = [(experimental release – spontaneous release) ÷ (maximum release – spontaneous release)] x 100%.

In this study we compared DC cross-primed CTLs with non cross-primed CTLs for cytotoxic lysis of SK 24 Mel and Ed 276 Bt target tumour cells. Further, in order to test specificity and safety against activating cytotoxic responses against normal target cells we used entirely autologous material, CTLs activated by DCs primed with autologous peptide extracted from skin fibroblasts and evaluated for cytotoxic activity against fibroblast targets.

Results:

A. Evaluation of tumour peptide extracts

In this pre-clinical investigation we had three primary endpoints. First we wanted to validate the consistent extraction of naturally processed peptides using the method of Protti et. al. [17], from early explant cultures generated from surgical excisions of gliomas and malignant melanomas. In this study we evaluated 8 different extracts. Each extract was evaluated by high pressure liquid chromatography (HPLC) and amino acid analysis by an independent laboratory; The Alberta Peptide Institute. The results of total peptide concentrations for the various preparations are displayed in **Table 2-1**. Confirmation of tumour peptide protein size was also confirmed by running samples on a TRIS/Tricine 10-20%.

B. Characterization of autologous dendritic cell isolation and maturation

The second endpoint we were concerned about was our ability to consistently generate mature dendritic cells from circulating CD 14+ monocytes. Using fluorescence immunohistochemistry in conjunction with confocal microscopy we were able to demonstrate dendritic cells with mature morphology as well as intense surface expression of characteristic molecules such as CD 83 – **Figure 2-1**. We used FACs analysis to demonstrate our ability to isolate immature DCs and subsequently generate mature DCs after stimulation with a maturing cytokine cocktail and tumour peptide. In **Figure 2-2** we observed that mature DCs indeed demonstrated profound increases in surface expression of CD 80, CD 83, CD 86, and MHC class II molecules. In the mature state no DC preparation displayed evidence of persistent CD 14 positivity. We also confirmed that the use of GM-CSF & IL-4 to generate DCs was in our hands the most effective in generating mature DC characteristics - **Figure 2-3**.

C. Evaluation of DC ability to activate cytotoxic T-lymphocytes

The third and most important endpoint of this investigation was to determine if peptide pulsed DCs using our methodology could activate CTLs. The protocol followed for activation of CTLs with peptide pulsed DCs is outlined in **Figure 2-4.**

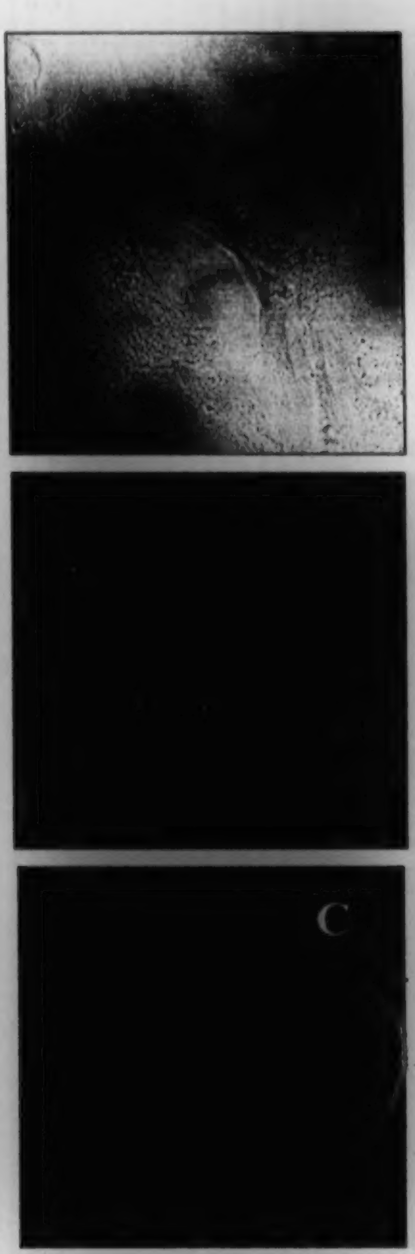

Figure 2-1 Microscopy Evaluation of Dendritic Cells

Microscopy evaluation of the morphology of dendritic cells generated from circulating peripheral blood CD14+ monocytes. **A**= immature dendritic cells. No fluorescent signal for the CD 83 could be detected so not shown. **B** = phase contrast image of typical mature dendritic cells. **C** = Fluorescent microscopy detecting strong surface expression of CD 83, a hallmark of dendritic cell maturation.

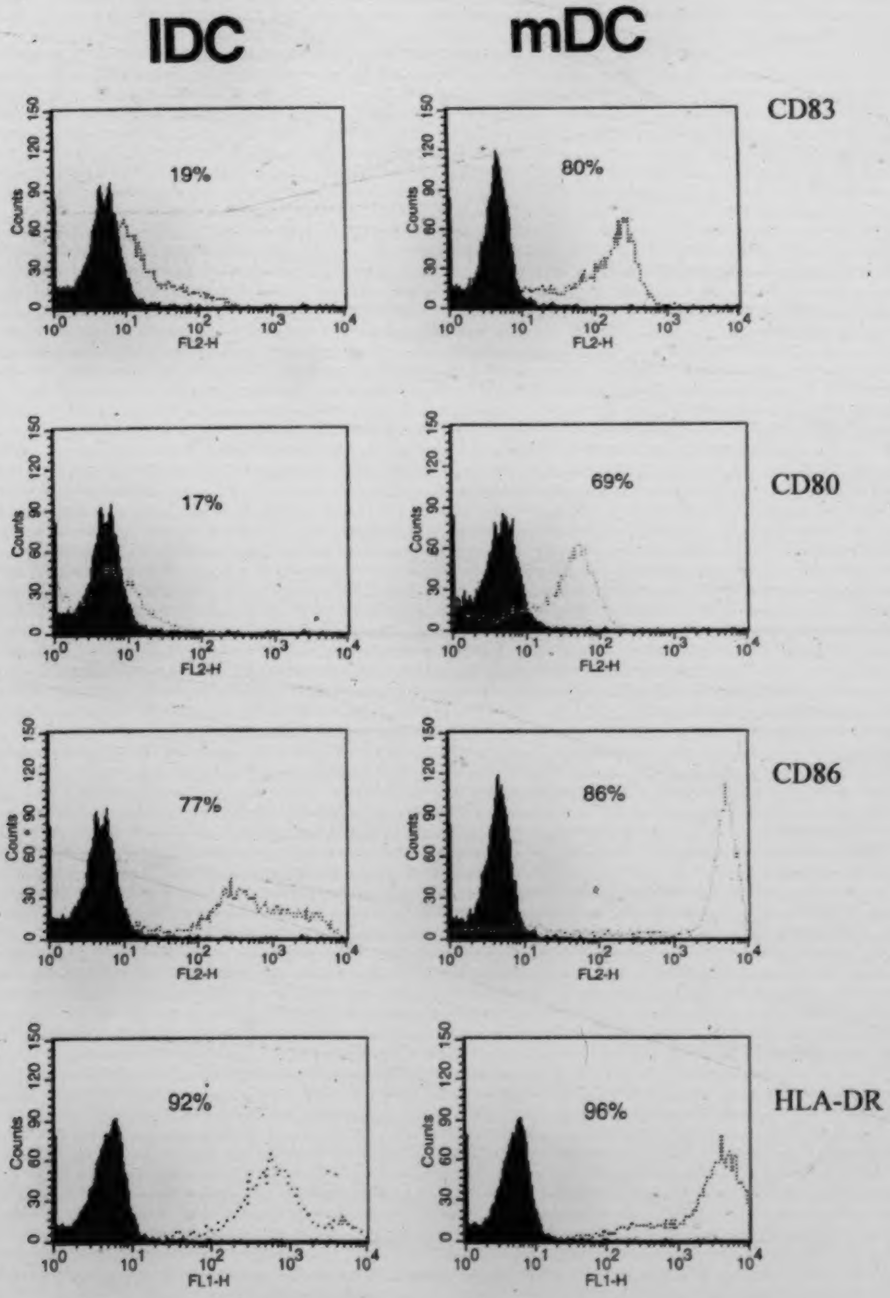

Figure 2-2 Dendritic Cell Surface Expression of Markers of Maturation

Evaluation of surface expression of CD 83, CD 80, CD 86, and MHC class II (HLA-DR) by immature and mature dendritic cells generated form peripheral blood circulating CD 14= monocytes using our standard protocol. The dendritic cells were evaluated using flow cytometry (FACS). **IDC**=immature DCs, **mDC**= mature DCs.

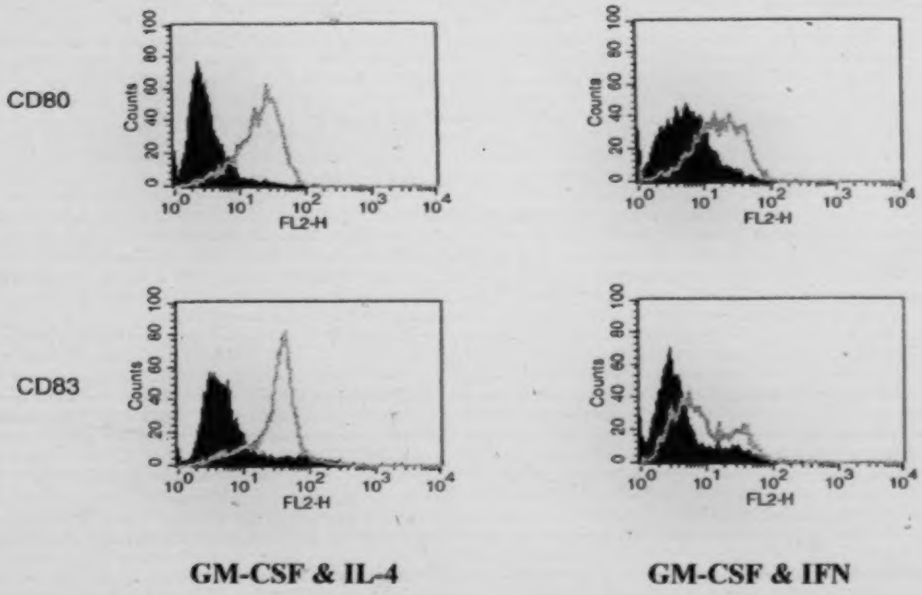

Figure 2-3 Comparison of Two Methods of DC Maturation

Evaluation of surface expression of CD 80 and CD 83, by mature dendritic cells generated form peripheral blood circulating CD 14= monocytes using our standard protocol using GM-CSF & IL-4 compared with use of GM-CSF & IFN to support maturation. The dendritic cells were evaluated using flow cytometry(FACS).

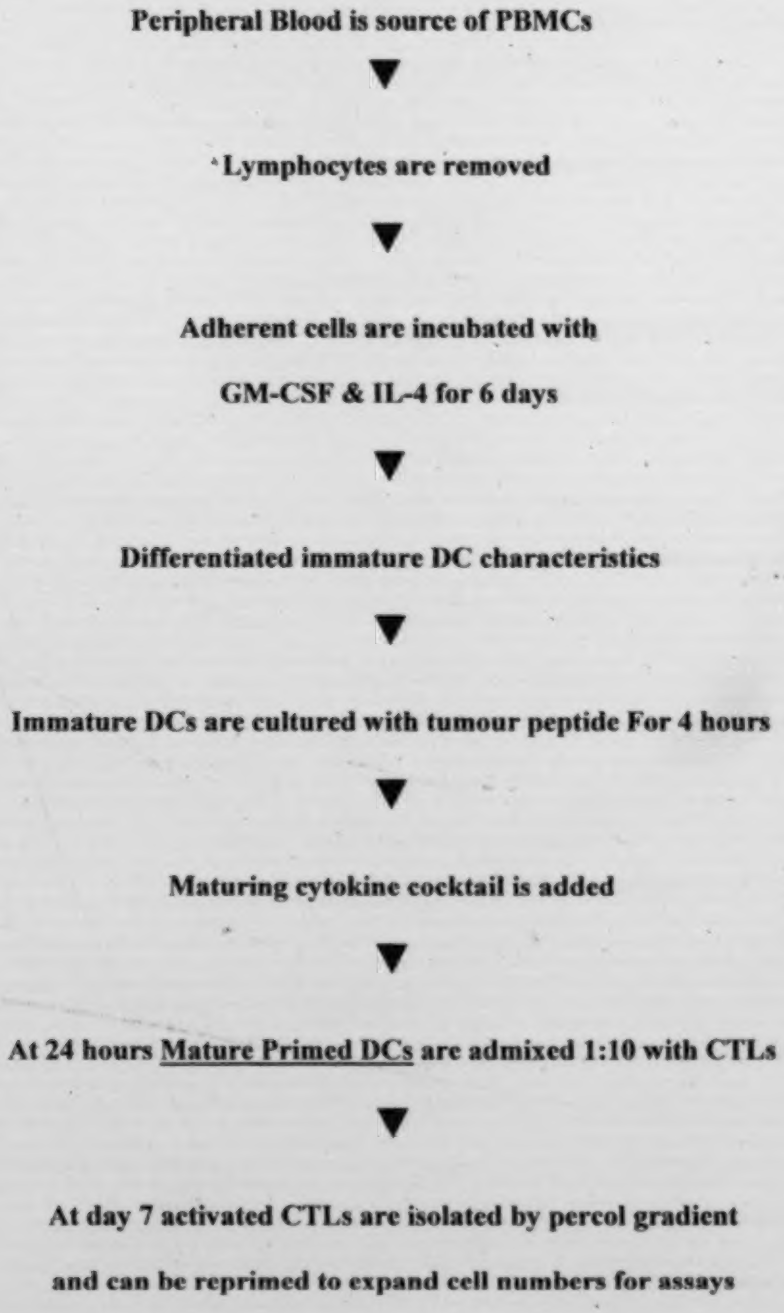
Peripheral Blood is source of PBMCs
Lymphocytes are removed
Adherent cells are incubated with GM-CSF & IL-4 for 6 days
Differentiated immature DC characteristics
Immature DCs are cultured with tumour peptide For 4 hours
Maturing cytokine cocktail is added
At 24 hours Mature Primed DCs are admixed 1:10 with CTLs
At day 7 activated CTLs are isolated by percol gradient and can be reprimed to expand cell numbers for assays

Figure 2-4 Flow chart depicting protocol sequence for generating mature peptide pulsed DCs and subsequent cross-priming of CTLs

C1. CTL Proliferation

We consistently observed that both HLA-A2 matched and autologous CTLs underwent rapid proliferation after admixture with peptide pulsed DCs. An example of this can be seen in **Figure 2-5**.

C2. Activated CTLs Secrete IFNγ

In addition to clonal expansion activated CTLs secrete IFNγ. In **Figures 2-6 & 2-7**, the strong IFNγ release by both HLA-A2 matched and autologous CTLs as a result of stimulation by peptide pulsed DCs is displayed. Importantly, in **Figure 2-6**, the ability to generate strong IFNγ release was sustained with consecutive repriming and did not show evidence of weakening. In **Figure 2-7,** it is interesting that the level of IFNγ response was strong in all subjects but 10 fold stronger for the autologous preparation for Ed 343 Mel.

C3. Evaluation of Activated CTLs with Cr^{51} Release Assay

Ultimately we were interested in the ability of activated CTLs to kill target tumour cells. In **Figures 2-8A & B** the results of Cr^{51} release assays are shown. Using HLA-A2 matched CTLs and DCs against SK 24 Mel, and Ed 276 Bt target cells up to 38% and 58% target cell lysis was observed respectively at a 1:20 target/effector cell ratio after only four hours. We also noted that for autologous DCs and CTLs activated

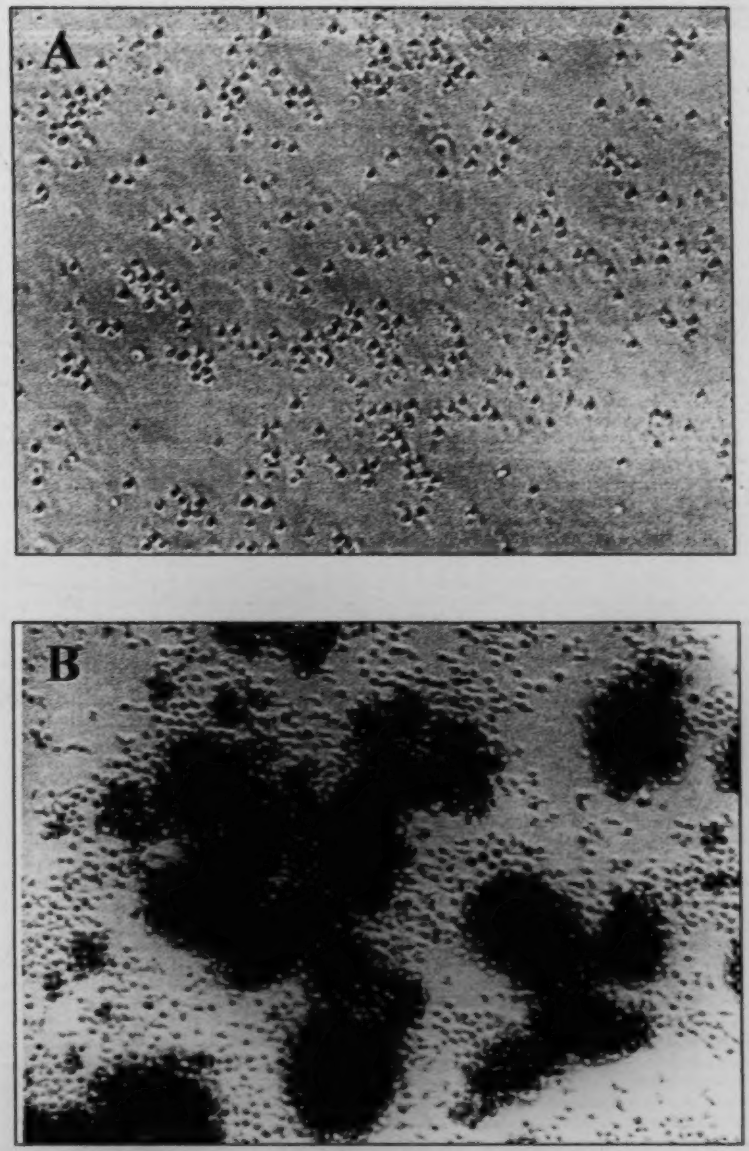

Figure 2-5 Clonal Expansion of Autologous CTLs

Light microscopy of autologous cytotoxic lymphocytes for Ed 326 Bt. **A** = 4 hours after admixture of autologous CD 8+ lymphocytes with autologous mature DCs that had been pulsed with autologous naturally processed tumour peptides. **B** = the same preparation at 48 hours after admixture showing significant clonal expansion of CTLs.

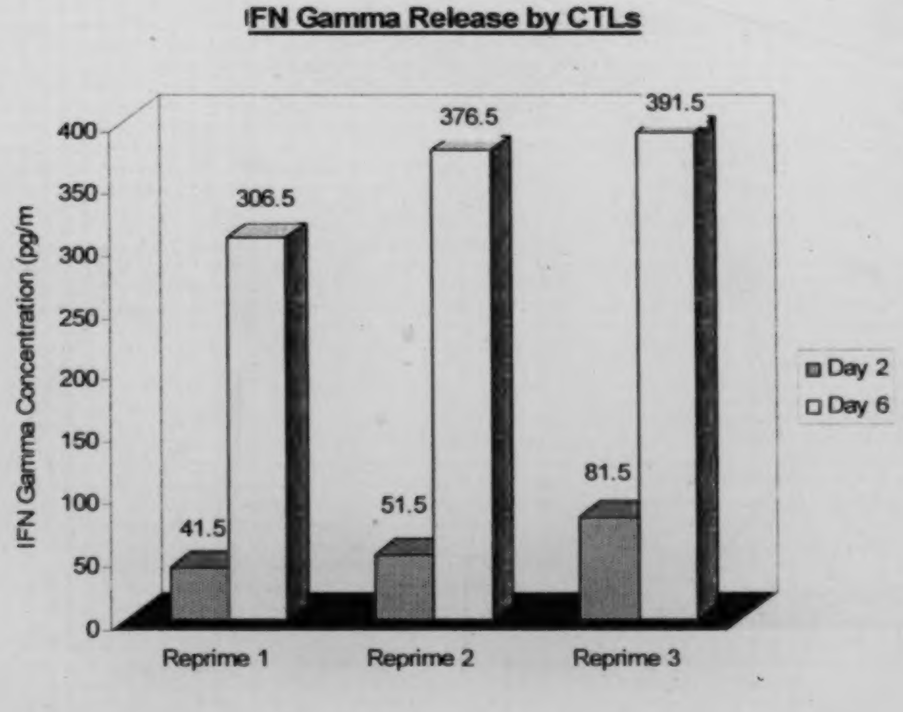

Figure 2-6 **IFNγ Release with Repeated CTL Repriming with HLA-A2 Matched Peptide Pulsed DCs.**

This graph depicts the IFNγ release by HLA-A2 matched CD 8+ lymphocytes *in vitro* after admixture with HLA-A2 mature DCs from the same donor that had been pulsed with naturally processed peptide from the melanoma tumour cell line SK 24 Mel. Culture media was collected and evaluated by ELISA assay for IFNγ on day 2 and day 6 after admixture. Repriming took place at one week intervals.

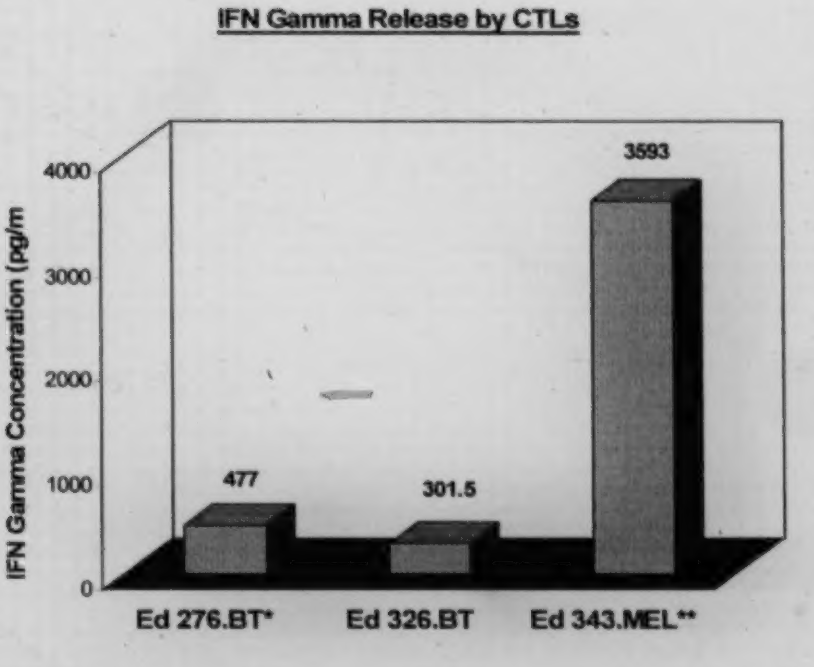

Figure 2-7 **IFNγ Release by CTLs Following Stimulation by DCs Pulsed with Early Explant Tumour Peptides.**

This graph depicts the IFNγ release by CD8+ lymphocytes *in vitro* after admixture with mature peptide pulsed DCs. Culture media samples were collected on day 6 after admixture and IFNγ levels detected by ELISA assay. * For Ed 276 Bt donor HLA-A2 matched CTLs and DCs were used. For Ed 326 Bt and Ed 343 Mel, autologous CTLs and DCs were used. ** For Ed 343 Mel unpulsed autologous DCs or mixed PBMCs did not induce relaease of detectable IFNγ.

A

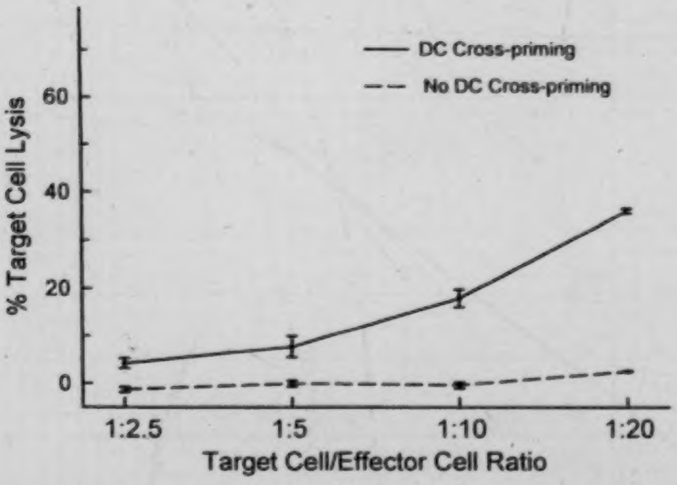

Figure 2-8A Cr^{51} release assay was used to detect SK 24 Mel target cell lysis by HLA-A2 matched CD8+ lymphocytes that were activated by mature HLA-A2 DCs pulsed with SK 24 Mel naturally processed tumour peptides. Results depicted are for an interval of 4 hours after effector and target admixture. Comparision is made between CTLs that had DC cross-priming vs no DC cross-priming.

B

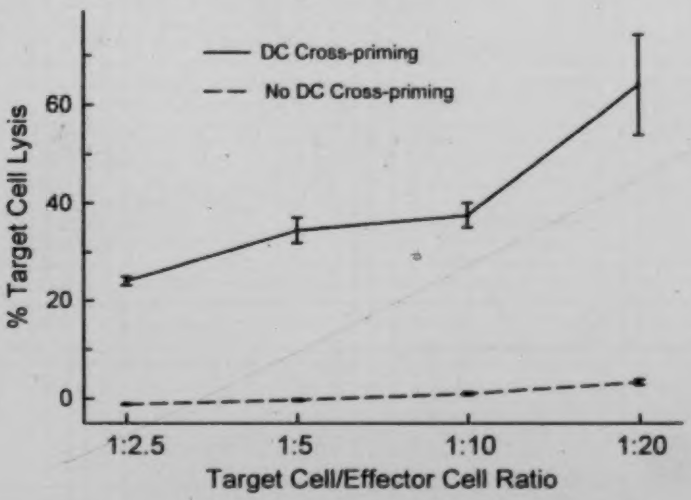

Figure 2-8B Cr^{51} release assay was used to detect Ed 276 Bt target cell lysis by HLA-A2 matched CD8+ lymphocytes that were activated by mature HLA-A2 DCs pulsed with Ed 276 Bt naturally processed tumour peptides. Results depicted are for an interval of 4 hours after effector and target admixture. Comparision is made between CTLs that had DC cross-priming vs no DC cross-priming.

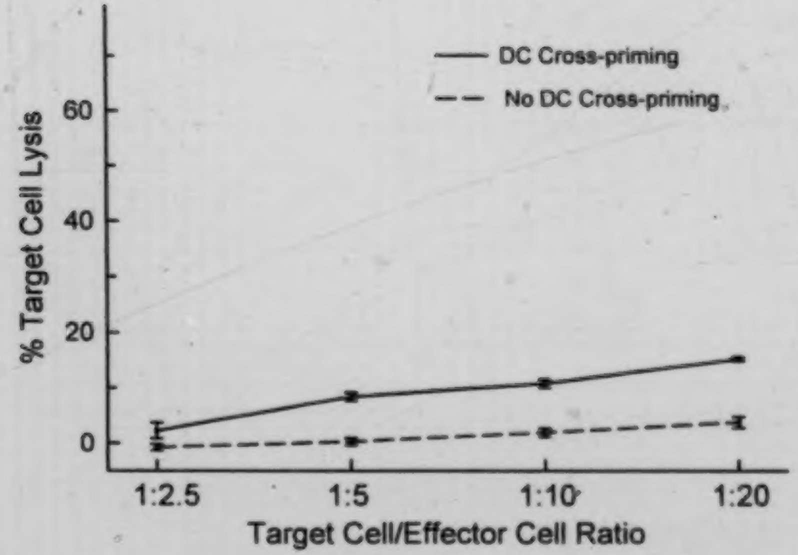

Figure 2-9 Cr^{51} release assay was used to detect human cultured fibroblast target cell lysis by autologous CD8+ lymphocytes that were activated by mature autologous DCs pulsed with naturally processed peptides from cultured fibroblast cells. Results depicted are for an interval of 4 hours after effector and target admixture. Comparision is made between CTLs that had DC cross-priming vs no DC cross-priming.

with peptides form autologous skin fibroblasts there was no significant activity against the fibroblast targets – **Figure 2-9.** This finding was very suggestive of the safety of this method against normal tissues.

Discussion:

The purpose of this pre-clinical study was to determine whether dendritic cells primed with naturally processed peptides extracted from glioma or melanoma tumour cells could induce cytotoxic T-lymphocyte activation, proliferation, and cytotoxicity against tumour cells. We have adapted the method initially created by Protti et.al [17]. The particular strength of using naturally processed peptides restricted to between 100 and 5,000 Daltons in size is the facilitation of peptide presentation in the context of MHC class I molecules, a requirement for engaging a strong Th1 immune response, without requiring additional processing by the DC. Furthermore, DCs primed with naturally processed peptide have clearly been demonstrated to induce specific cytotoxic activity against a wide range of antigen epitopes including tumour antigens that as yet have not been individually characterized. We confirm in this study the ability to consistently generate naturally processed peptides from early explant cultures of both malignant melanoma and glioma tumour cells. In our hands we found that use of the combination of IL1β, IL-6, TNFα, and PGE_2 was the most consistent maturing cytokine cocktail for the generation of mature primed DCs from patients harbouring gliomas and malignant melanomas. We also confirmed that autologous and HLA-A2 matched CTLs could be cross-primed/activated by naturally processed tumour peptide by mature DCs, as evidenced *in vitro* CTL clonal expansion and CTL IFNγ secretion. Repeated exposure to tumour peptide in the context of mature DCs did not *in vitro* appear to cause tolerance. Finally, autologous or HLA-A2 matched DCs primed with the array of naturally processed tumour peptides from malignant melanomas and gliomas could induce CTLs *in vitro* to kill target tumour cells from which the tumour peptides originated. On the basis of this study we propose that generation of autologous dendritic cells primed with autologous naturally processed tumour peptide is a viable strategy for an adoptive immunotherapy vaccination for patients diagnosed with gliomas or malignant melanomas.

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CHAPTER 3

Progress Report of Autologous Dendritic Cell Vaccine Trial for Patients with Malignant Melanoma

3

Introduction:

It is well recognized that the worldwide incidence of melanoma is increasing. In the United States the lifetime risk of having melanoma is now 1: 75. Though, educational initiatives and screening measures for early detection may provide some benefit in terms of early access to definitive treatment of low grade disease, the outlook for patients harbouring American Joint Committee on Cancer Staging (AJCC) grade III or IV disease is very poor with median survival times of 7 to 9 months[1].

Melanoma has a strong predilection to metastasize to the central nervous system (CNS). In autopsy series cerebral metastases were found in up to 75% of melanoma patients[2, 3]. CNS metastases were found to be the cause of death in about 1/3 of patients[4]. Sampson et. al. observed that the median survival of patients with metastases to the brain is less than three months[5]. In a large retrospective study involving 147 patients with cerebral metastases, the combination of surgical resection followed by whole brain radiation therapy (WBRT) resulted in a median survival of only 8.5 months[6]. The use of radiosurgery offers similar median survival rates between 7 and 10.4 months[7-9]. Chemotherapy protocols also offer poor prognosis for treatment of stage IV disease with best median survival outcomes between 6.9 and 10.7 months as reviewed recently by Atkins et. al[10].

In a retrospective review of 777 cases of AJCC stage I and II primary cutaneous melanoma focusing on those patients with a minimum follow up of 10 years it was found by multivariate analysis that the presence of tumour infiltrating lymphocytes was a significant positive prognostic factor[11]. The observation that the response of the host immune system to tumour affects the course of the disease coupled with the fact that currently available surgical, radiation, and chemotherapeutic interventions are of limited efficacy has made immunotherapy an attractive treatment consideration.

Immunotherapy in general involves two main strategies; modulation of a specific tumour's cytokine and growth factor profile, and manipulation of immune response cells to bring about a specific cytotoxic targeting of tumour cells. In order to generate a robust and specific cellular immune response three interdependent interactions must occur. First, T-lymphocyte receptors (TCR) must recognize and bind to antigen in the context of major histocompatability complex (MHC). Second, co-stimulatory molecules such as T-lymphocyte membrane molecules CD28 (cluster of differentiation 28) or LFA-1 (lymphocyte function-associated antigen-1) must interact with antigen presenting cell (APC) or target cell ligands B7-1 or ICAM-1 (intracellular adhesion molecule-1) respectively. Both successful antigen recognition and co-stimulatory molecule binding leads to signal transduction that results in T- lymphocyte generation of the third and crucial event for cytotoxic immune response – generation and secretion of interleukin-2 (IL-2).

Melanomas like most tumours have evolved multiple strategies to avoid immune detection and truncate immune responses. In brief, melanoma cells avoid immune surveillance by reducing surface antigen presentation in the context of MHC[12, 13]. They also generate molecules in the tumour microenvironment that can dramatically impair the immune response of T-lymphocytes and APCs[14, 15]. As an example, transforming growth factor beta (TGF-β) is highly expressed by melanomas, and is associated with immune cell suppression and decreased MHC II antigen presentation[16]. Another example is secretion of the cytokine IL-6 which interferes with generation of an interferon gamma (IFNγ) mediated Th1 cytotoxic response[17]. IL-10 also produced by melanoma cells has been observed to inhibit APC stimulation of T-lymphocytes,[18, 19] and is recognized to contribute to immune tolerance[20]. Finally, some melanoma cells may ultimately avoid T-lymphocyte induced cytotoxicity by having evolved anti-apoptotic strategies[21].

Dendritic cells (DCs), first described by R. Steinman, are professional APCs that are the most powerful initiators and modulators of immune responses[22]. DCs have the all important characteristics of efficient capture and processing of antigen, robust membrane expression of MHC-antigen complexes, and provision of both surface expression of co-stimulatory molecules and secretion of stimulatory cytokines[23]. Because of these all important characteristics for generation of both active effector immune responses and immunologic memory, DCs have become the focus of interest for generating adoptive immunotherapy stategies against cancers. On the basis of pre-clinical investigations of murine models of melanoma[24, 25], and *in vitro* studies demonstrating specific anti-tumour responses[26], human clinical trials have been pursued. The first clinical application of dendritic cell therapy was against lymphoma[27]. Shortly thereafter, F. Nestle et. al. conducted the first DC vaccination trial for melanoma patients demonstrating both safety and antigen specific immunity[28]. The promise of these early clinical trials has encouraged the pursuit of refining optimal therapeutic regimens for DC based immunotherapies for cancer.

In this trial we investigate the safety, tumour specific immune response, and clinical efficacy of an autologous dendritic cell vaccine combined with a unique comprehensive immunoadjuvant regimen. We report our results for the first four patients in a phase I, Health Canada approved, trial for the treatment of AJCC stage III & IV melanoma. In particular we were interested in the treatment of patients with CNS involvement. All patients were vaccinated on an outpatient basis with autologous CD14+ monocyte derived DCs that had been primed and activated *ex vivo* with naturally processed peptides derived form early explant cultures of autologous surgically resected melanoma tumours. Throughout the vaccination protocol patients received immunoadjuvant treatment consisting of IL2, granulocyte macrophage – colony stimulating factor (GM-CSF), IFNα2b, all-trans retinoic acid, and Celecoxib. At this interval we make three observations. Firstly, no patient has had a toxic response greater than grade 1 (mild), according to the National Cancer Institute of Canada Clinical Trials Group expanded common toxicity criteria. Secondly, all patients for whom pathology specimens were available after starting the vaccination protocol (3/4) demonstrated significant immune cell infiltration of their tumours. Finally, those patients who harboured intracranial metastases had a mean survival of 17 months (range 8-29) from the time of diagnosis in contrast to an expected survival of approximately 3 months[5].

Methods:

Prior to commencing clinical investigations on patients the trial protocol was reviewed and approved by the University of Alberta Research Ethics Board. In addition, the protocol entitled A phase I study of autologous dendritic cell vaccination and immunoadjuvant therapy for malignant gliomas and medically refractory melanomas (file number 9427-U0180-54C), was approved for patient enrolment and treatment by Health Canada – Health Protection Branch.

Patient eligibility

In this study we present the first four patients to participate in the vaccination trial. Trial recruitment of patients with high grade astrocytomas or malignant melanomas is ongoing. All patients in this preliminary report haboured progressive AJCC stage III-IV melanoma and were referred by a medical oncologist. Inclusion into the trial required: histologically confirmed melanoma, documented progression despite treatment, a Karnofsky Performance Scale Score

> 60, a surgical biopsy from each patient and subsequent generation of a tumour explant culture. Exclusion criteria included: associated significant non-malignant disease, such as infection with human immunodeficiency virus (HIV), chronic/active hepatitis, liver cirrhosis, renal failure, and autoimmune disease, prior immunotherapy or chemotherapy within previous 6 weeks, pregnancy, and sensitivity precluding compliance with the immunoadjuvant medications for the trial. All patients gave written consent after two separate trial information meetings conducted by different study members.

Autologous tumour culture and preparation of peptide

The procedures for the preparation of the vaccine peptide have been extensively detailed in the methods and materials section of chapter 2 of this thesis. In brief, melanoma tumour cells were dissociated from fresh surgical specimens. Isolation of cells involved mechanical and enzyme treatment followed by isolation on a Ficoll-Paque Plus (Amersham) density gradient. The tumour cells were plated in culture flasks and expanded in complete RPMI (Rosewell Park Memorial Institute) 1640 culture media supplemented with 2mM L-glutamine (Invitrogen), 100μM sodium pyruvate, and 10% fetal bovine serum. The cells were incubated and humidified at 37°C and 5% $C0_2$.

Before a tumour peptide extract was generated from early explant culture cells, melanoma diagnosis was confirmed by the final clinical pathology report of the originating surgical specimen as well as by immunohistochemical studies of the culture cells using: mouse anti-human fibroblast antibodies (Dako), mouse anti-human Melan-A (Novo Castra NCL), and mouse anti-human malignant melanoma HMB45 (Biogenix).

We utilized the method of isolating naturally processed tumour peptides as previously described by MP Protti et. al.[26]. Approximately 1 x 10^8 cultured tumour cells were used to generate each peptide extract. The cells were thoroughly rinsed to remove all exogenous proteins. The cells were then lysed with four freeze-thaw cycles in the presence of 2mM TRIS/HCl, 10mM NaCl, and 0.1% trifluoroacetic acid. After centrifugation the resulting supernatant was placed in a 100 Dalton MWCO dialysis tube (Spectra/Por) and submerged in a phosphate buffered solution (PBS) bath for a minimum of 16 hours to remove peptides smaller than 100 Daltons. The dialysate was then subjected to centrifuge filtration using a 5,000 NWML Ultrafree®-Cl filter (Amicon Millipore). The extract now containing peptides between 5,000 and 100 Daltons in size was filter sterilized. A 30 μL sample of each peptide extract was submitted to an independent laboratory (The Alberta Peptide Institute) for evaluation of peptide concentration based on calculation of the analyzed amino acid content. Samples were also submitted to the local Provincial Laboratory for confirmation of sterility, mycology testing and mycoplasma evaluation. The remainder of the extract was aliquoted and stored at -80°C until required for the priming of autologous dendritic cells and for inclusion in the vaccine bolus mixture.

Preparation of autologous dendritic cells

The method of generating dendritic cells from PBMCs (peripheral blood, CD14+ monocytes) is detailed in chapter 2 of this thesis. Following the techniques developed by Steinman and Lanzavecchia [29, 30] we generated autologous dendritic cells for each patient's vaccine. In brief, each patient underwent a phlebotomy (400 cc of whole blood) by the University of Alberta Autologous Blood Donation Team. Whole blood was diluted 1:1 with Hank's Buffered saline solution (HBSS) and then layered over Ficoll-Paque®Plus. PBMCs were isolated form the gradient interface after centrifugation. The cells were suspended in RPMI 1640 media containing 4mM L- glutamine and 2% human AB+ heat inactivated serum (Bio-Whittaker). Cells were plated on 6 well tissue culture plates (Costar Corning) at a concentration of 7 x 10^6 cells/mL and incubated for 2 hours. After 2 hours non- adherent cells were removed and the adherent, CD14+ monocytes were then maintained in complete RPMI culture media supplemented with 1,000 IU/mL granulocyte monocyte-colony stimulating factor (GM-CSF)(Immunex), 500 IU/mL interleukin 4 (IL4)(CellGenix), and 5% human AB+ serum. Cytokines were refreshed every 48 hours. On day 6 the resulting immature non adherent dendritic cells were primed with autologous tumour peptide extract.

The immature DCs were harvested and incubated for 4 hours in 0.5 mL of media with autologous tumour peptide extract at a concentration of 100 μg/ mL.

After 4 hours a maturing cytokine cocktail including: Interleukin 1β (CellGenix) at 20ng/mL, Interleukin 6 (IL-6) (CellGenix) at 500 IU/mL, Tumour necrosis factor α (CellGenix) at 20 ng/mL, and Prostaglandin E_2 (Amersham Pharmacia Europe) at 2μg/mL was added to the peptide pulsed DCs to induce full maturation. The cells were incubated for 24 hours in 2mL of supplemented media at 37°C with 5% CO_2. After 24 hours the mature peptide-primed DCs were washed once with HBSS. A sample was submitted to the local Provincial Laboratory for confirmation of sterility, mycology testing and mycoplasma evaluation. Aliquotes of the remaining DCs were made. 2×10^6 cells/mL were suspended in 1mL of freezing media consisting of 10% v/v DMSO, 5% v/v glucose, in human AB+ serum. Cells were cooled in a cryofreezing container (Nalge Nunc) for 24 hours at -80°C and then transferred to storage in liquid nitrogen until required for vaccination.

Final vaccine preparation and dose administration

Prior to any patient injection the trial vaccination records were reviewed to confirm that safety testing of all components of the injectable had been confirmed sterile by independent testing through the local University of Alberta Provincial laboratory site. On the day of patient injection a vial of cryopreserved, autologous, peptide-primed DCs were thawed quickly in a 56°C water bath and transferred to a centrifuge tube containing isotonic patient grade saline (Astra Zeneca). A total of three washes in saline were performed and viable cells counted so that 1 x 10^6 DCs were mixed with 100 μg of autologous tumour peptide extract, and 50 μg of Keyhole Limpet Hemocyanin (KLH) (Intracell). The mixture was brought to a final volume of 200 μL with isotonic saline and drawn into a 1cc syringe with a 25g – 5/8 inch needle. The syringe was labeled and transferred immediately from the laboratory to the University of Alberta Hospital ultrasound suite where a qualified radiologist assisted in the localization and injection of 100 μL of vaccine into lymph nodes under ultrasound guidance. The remaining 100 μL of vaccine was injected intradermally. Patients were observed and vital signs monitored for 3 hours following vaccine injections. Patients were contacted by phone daily for two days to document any adverse reactions or symptoms.

We utilized 1 x 10^6 autologous DCs on the basis of previously established safety and suggested efficacy in other vaccination trials[31, 32]. We used an autologous peptide extract bolus of 100 μg based on similarly established safety as well as observations in our own preclinical studies (chapter 2) and those of others, that this concentration could establish DC peptide priming[33]. KLH is a well established adjuvant in vaccine composition that is in general used for evaluation of delayed type hypersensitivity reactions. We injected our vaccine into lymph nodes and intradermally on the basis of work by Fong et. al. [34]. The cervical lymph nodes constitiute the normal lymphatic drainage route for the brain. By injecting within the node and in the perinodal region we avoid the immediately suppressive milieu of the tumour itself and situate the primed activated autologous DCs in closest proximity populations of immature effector T-lymphocytes which could potentially undergo cross priming.

Treatment protocol

Patients received their intranodal and intradermal vaccine injections at 2-3 week intervals. For patients with intracranial lesions the nodal injections were given in the cervical lymph nodes with ultrasound guidance. For patients with peripheral lesions the nodal injections were given in the inguinal lymph nodes with ultrasound guidance. **Figure 3-1** outlines the trial protocol. **Figures 3-2** to **3-5** indicate the actual timelines for each trial patient presented in this study.

The immunoadjuvant therapy consisted of the following: GM-CSF (250μg/m^2/day) was given for 5 days pre- and post- injections. IFN α2b (3 MIU/m^2/day) and IL-2 (2 MIU/m^2/day) were both started 5 days prior to the first vaccination and continued until 1 month after the 3rd vaccination. Oral all-trans retinoic acid (22mg/m^2 b.i.d.) was started 5 days before the first vaccination and continued until 1 month after the last injection. The cyclooxygenase-2 enzyme (COX-2) inhibitor, Celecoxib (200 mg BID) was started 5 days prior to the first vaccination and continued for the entire year while the patient was on-trial.

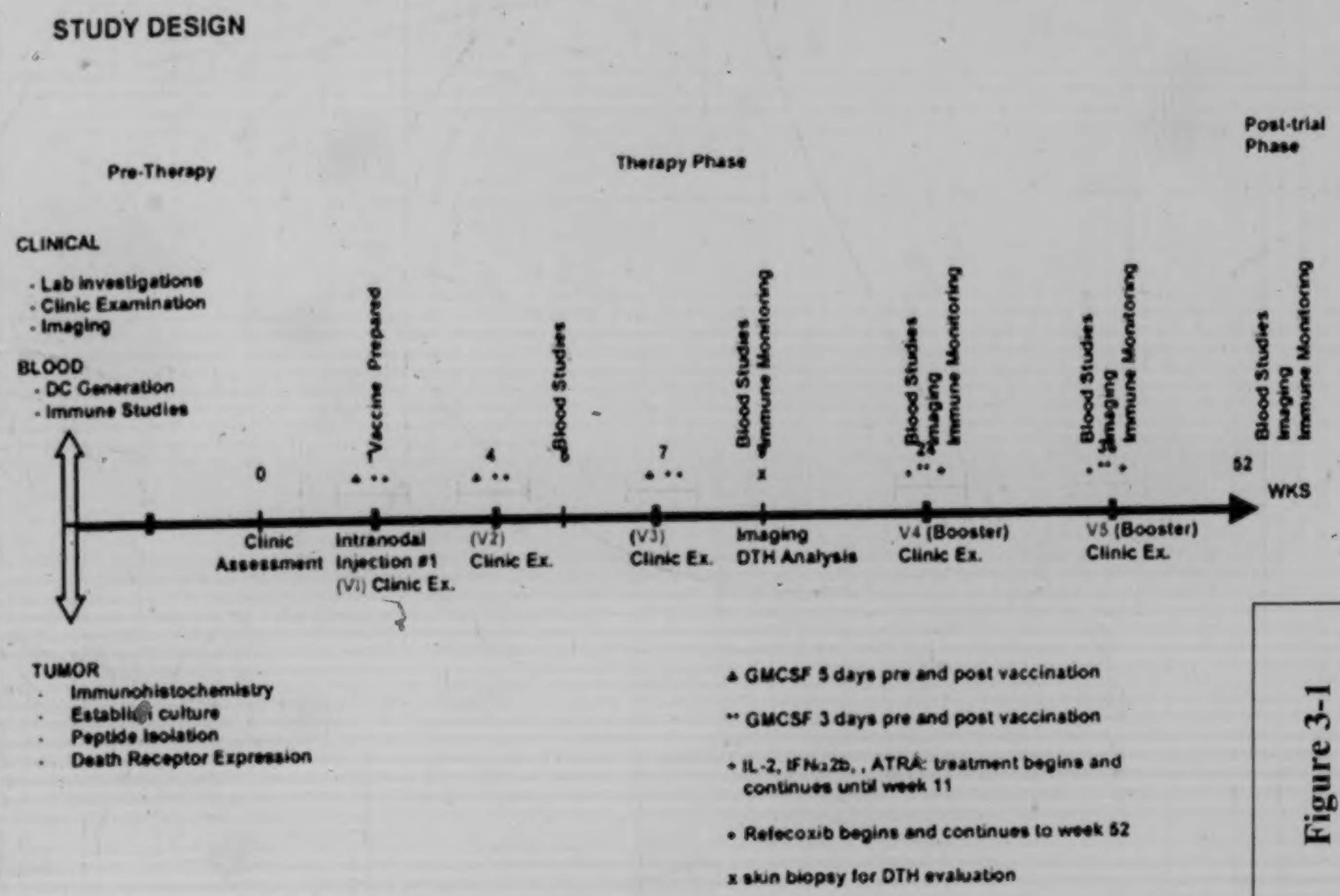

STUDY DESIGN
Pre-Therapy
Therapy Phase
Post-trial Phase
CLINICAL
- Lab investigations
- Clinic Examination
- Imaging
BLOOD
- DC Generation
- Immune Studies
TUMOR
Immunohistochemistry
Establish culture
Peptide Isolation
Death Receptor Expression
0
Clinic Assessment
Vaccine Prepared
Intranodal Injection #1
(VI) Clinic Ex.
4
(V2)
Clinic Ex.
Blood Studies
7
(V3)
Clinic Ex.
Blood Studies
Immune Monitoring
Imaging
DTH Analysis
Blood Studies
Imaging
Immune Monitoring
V4 (Booster)
Clinic Ex.
Blood Studies
Imaging
Immune Monitoring
V5 (Booster)
Clinic Ex.
52
WKS
Blood Studies
Imaging
Immune Monitoring
▲ GMCSF 5 days pre and post vaccination
** GMCSF 3 days pre and post vaccination
+ IL-2, IFNα2b, , ATRA: treatment begins and continues until week 11
• Refecoxib begins and continues to week 52
x skin biopsy for DTH evaluation

Figure 3-1

FIGURE 3-2.

Trial timeline for EPIC 001

- **1982** Dx melanoma left calf.
- **1987** mets to left groin resected.
- **1996** left groin dissection and treatment with IFN.
- **1998** stage II breast Ca identified and treated with Tamoxifen and radiation.
- **1999** repeat left groin dissection for melanoma recurrence.
- **2000** isolated left leg perfusion chemotherapy.
- **2002** mediastinal melanoma positive nodes biopsy, chemotherapy with DTIC, IL-2, IFNα2b.
- **April 2003** 1cm left parietal brain met identified. Treated with γ- knife radiosurgery in San Diego.

- V1 Sept 17/03
- V2 Oct 10/03
- V3 Oct 29/03

- New onset left leg symptoms

- V4 Jan 28/04
- New brain mets and lump on left shoulder

- Mar 10/04 progression of brain mets, new scalp lesions

- Apr 7/04 7 new scalp lesions, 9 new skin lesions

- Apr 16/04 new onset seizures

- V5 Apr 28/04
- V6 May 5/04
- V7 May 12/04
- V8 May 21/04
- V9 Jun 3/04
- V10 Jun 7/04
- V11 Jun 21/04

- **DECEASED JUN 27/04**

- Feb 17/04 surgery to resect intrathecal metastasis T12-L1.
- Feb19-Mar 30 5 cycles Temozolamide.
- Mar 16/04 WBRT 3000cGy 10 fractions, spinal 1500cGy 5 fractions.

- May 5/04 radiation for tongue lesion.

FIGURE 3-3.

Trial timeline for EPIC 004

- **Nov 2002** Dx of nevus on left shoulder. Excisional biopsy.
- **Nov 2003** recurrence in previous scar Dx of melanoma.
- **Dec 2003** deep resection of left scapular recurrence. Left axillary lymph nodes positive.
- **Jan 2004** Interferon treatment started.
- **Jul 2004** metastases to left ilium.
- **Aug-Sept 2004** 10 fractions of radiation to left hemipelvis.

- V1 Sept 1/04
- V2 Sept 15/04
- V3 Sept 29/04
- V4 Oct 6/04
- Headaches developed

- V5 Oct 27/04
- Right temporal lobe brain met
- V6 Nov 11/04

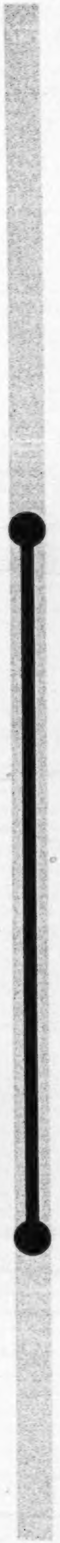
- Left parietal/occipital lobe brain met

- Nov 4&8/04 surgical resection of 3.5cm right temporal lobe met.
- Dec 20-Jan 12, WBRT
- Jan 19/05 Stereotactic radiosurgery for left occipital brain met

- **DECEASED JUL 12/05**

FIGURE 3-4.

Trial timeline for EPIC 006



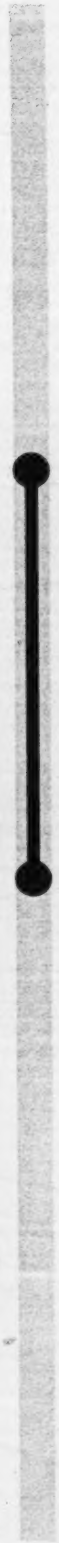
- **1997** Dx melanoma left face cheek. Excisional biopsy
- **1999** recurrence of left cheek tumour and involvement of cervical lymph nodes. Treated with focal radiation.
- **Jun 2000** left lung apex met. Surgical resection.
- **Aug 2003** left frontal brain met. Gross total surgical excision followed by WBRT 2500 cGy in 10 fractions.
- V1 Jul 14/04
- V2 Aug 8/04
- V3 Oct 20/04
- V4 Nov 10/04
- Right temporal lobe met
- V5 Nov 24/04
- V6 Dec 8/04
- Focal seizures affecting left leg
- Apr 05 8 mets throughout brain
- Aug 20/05 MRI shows progression of brain mets
- **DECEASED DEC 16/05**

- Nov 04, stereotactic radiosurgery to met. 1800 cGy

- May 04, 4 mets treated with stereotactic radiosurgery 1800 cGy each
- Jul 04 started low dose temozolamide and decadron

FIGURE 3-5.

Trial timeline for EPIC 007



- **Nov 2000** Dx melanoma left ankle. Excisional biopsy.

- **Apr 2001** left inguinal nodes positive at resection.

- **Dec 2001** 4 lesions over left leg surgically removed followed by isolated limb perfusion chemotherapy.

- **Jan 2003** recurrence at left ankle. Isolated left leg perfusion chemotherapy.

V1	Nov 25/04
V2	Dec 15/04
V3	Jan 5/05
V4	Jan 26/05
V5	Feb 23/05
V6	Mar 23/05

- Jul 05, small bruise right arm noted in clinic.

- Sept 19/05, surgical removal right arm, and superficial left chest wall metastases

- Jan 3/06, excisional biopsy of right buttock met and positive right axillary node.

- Mar 3/06, biopsy left axillary node positive.

- Mar 23/06, resection of left ankle recurrence with skin grafting.

- Aug 16/06, biopsy of right groin nodes positive.

- July '07 Patient still alive

Clinical evaluations

In addition to the physical evaluation of patients at and post-vaccination as indicated before for adverse reactions, several laboratory tests were performed at each vaccination follow-up clinic visit. Blood was evaluated for the following parameters: CBC with leukocyte differential, INR, PTT, glucose, urea, creatinine, serum electrolytes including calcium, SGOT, SGPT, LDH, Alkaline phosphatase, amylase, lipase, total protein, albumin, TSH, Free T3 and T4. Urine was evaluated for presence of blood, protein, and glucose. All blood and urine evaluations were performed by the Department of Laboratory Medicine at the University of Alberta Hospital according to their established clinical protocols.

Evaluation of immune responses

A. Mixed Lymphocyte Proliferation

Blood samples were taken from patients by a registered phlebotomist before vaccination and at intervals as indicated in the results section. Mixed lymphocytes were isolated from the whole blood using a standard Ficoll gradient protocol. In brief, the blood samples were diluted 1:1 with Hank's balanced salt solution (HBSS). The resultant mixture was layered over Ficoll-Paque Plus (20ml of diluted blood over 15ml of Ficoll-Paque Plus). After centrifugation the peripheral lymphocytes isolated in the gradient interface layer were harvested and washed three times in HBSS. The mixed lymphocytes were then counted and plated in 96 well (Corning Costar) culture plates at 1 x 10^5 cells per well in 100 µL of RPMI media supplemented with 10% horse serum and 20U/mL of IL-2. The mixed lymphocytes were separated into three treatment groups: peripheral lymphocytes only, lymphocytes with 25µg/ml KLH added, and lymphocytes with 100 µg/ml autologous tumour peptide added. H^3-thymidine was added at 1µC per well. After 96 hours the supernatant was removed and cells resuspended in scintillation media. Radiation counts as an indicator of cellular proliferation was performed on a Beckman LS β-radiation counter.

B. *IFNγ Secretion*

Production of IFN-γ by PBMCs isolated from trial patients was determined using an enzyme-linked immunosorbent assay (ELISA) Quantikine kit (R & D Systems, Minneapolis, MN) according to the manufacturer's instruction. In brief, 1x 10^5 PBMCs harvested from patients before vaccination, and following vaccination treatment were evaluated. The PBMCs alone and peptide-pulsed autologous mDC (1x10^4) were incubated for 16 hrs at 37°C in 96-well plate. PBMCs alone, PBMCs with addition of KLH, and PBMCs with additional tumour lysate were evaluated. 50 uL of supernatant was harvested at 16 hours and assayed for IFNγ production. The amount of IFN-γ production was calculated based on the standardized curve provided for this assay.

C. *Cr^{51} Release Assay*

Cytotoxic T-cell activity was evaluated using a standard chromium release assay. In brief, archived pre-vaccination and post-vaccination PBMCs (4-5 $\times 10^6$ cells) collected from patients as per the immunization protocol schedule, were thawed and cultured in RPMI 1640 complete medium (10 % human serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 25 mM HEPES, and 100 IU/mL penicillin and 100ug/mL streptomycin). Autologous cultured tumour cells were incubated with 100uCi Na_2 [Cr^{51}] O_4 (GE Amersham, Buck, United Kindom) for 45 min at 37°C. After extensive washes with RPMI 1640, the labeled autologous tumour cells (target cells) were admixed with autologous PBMCs (effector cells) at an effector to target cell ratio of 50:1; 25:1; 12.5:1 and incubated in RPMI complete medium in round-bottom 96-well plate. After a 4 hour incubation, a 100uL sample of the supernatant was collected and its radioactivity measured. The specific percent cytotoxicity was calculated based on the following formula:

% specific release = [(experimental release – spontaneous release) ÷ (maximum release – spontaneous release)] x 100%.

D. *Patient Serum Profiling*

Blood samples drawn for the clinical parameters detailed previously were also submitted for evaluation of erythrocyte sedimentation rate (ESR) and serum concentration of; C-reactive protein (CRP), lactate dehydrogenase (LDH), S100β, and IgG immunoglobulins. Immunoglobulin levels, ESR, CRP, and LDH evaluations were performed by the Department of Laboratory Medicine at the University of Alberta Hospital.

For S100β evaluation serum sample drawn concurrently with the other blood samples was processed in our laboratory as follows. 5 mL of patient blood was collected in a non-heparinized vacutube using standard phlebotomy technique. The blood was immediately transferred to our laboratory where samples were centrifuged at 2000 rpm for 10 minutes to separate cells from the serum. The serum supernatant was aliquoted into cryotubes and stored at -80°C for later batch analysis. Serum S100β concentrations were quantified using S100β ELISA kit manufactured by Syn-X Pharma. The kit employs sandwich ELISA technique with microplated well coated with murine monoclonal anti-S100 antibody. The detection antibody is polyclonal rabbit anti-S100. The assay is further developed with polyclonal goat anti-rabbit IgG labeled with horseradish peroxidase. The assay was performed according to the manufacturer's directions. Colour detection for quantification purposes was measured at 450nm absorbance using a Bio-Rad model 680 photo spectrometer.

Results:

A. *Vaccine safety*

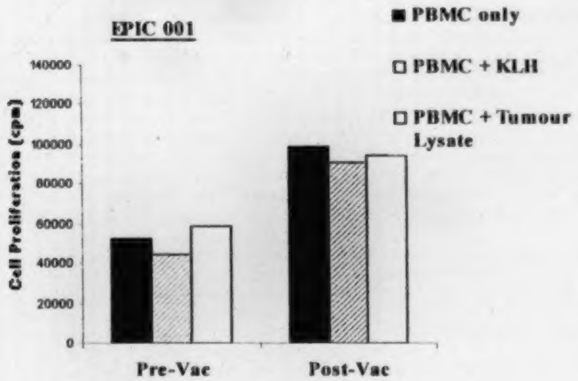
The primary endpoint of this phase I trial was to determine the safety of this vaccination and immunoadjuvant protocol. None of the patients had an adverse effect of the vaccination ≥ grade 1 (mild) according to the National Cancer Institute of Canada Clinical Trials Group expanded common toxicity criteria. All patients reported mild flu like symptoms for 24-48 hours after vaccination, described as mild fever combined with general malaise or body ache. No patient developed a hypersensitivity wound or reaction at the injection site. Furthermore, no systemic toxicity was identified in laboratory test of renal, or hematologic function. One patient, EPIC 001, had an isolated spurious elevation of liver enzymes noted at V3 (vaccine injection 3) which completely resolved at repeat testing within 1 week. This finding was not associated with clinical symptoms. In addition, no patient developed a non-specific cutaneous or neurologic toxicity.

B. *Immune Response to Vaccination*

The secondary endpoints of this study were induction of specific anti-tumour immune response, and increased patient survival. We determined development of an immune response by evaluating: patient lymphocyte activity, expression of certain serum markers, and pathology specimens.

B1. Lymphocyte Activity

We evaluated the first two patients in the trial, EPIC 001 and EPIC 004, for lymphocytic activity both pre- and post- vaccination. **Figure 3-6** demonstrates that both EPIC 001 and EPIC 004 developed increased mixed lymphocyte proliferation following vaccination. It should be noted that for EPIC 001 no differences were noted in proliferation depending on the type of *in vitro* stimulation provided. For EPIC 004, following a decrease in proliferative response after the first vaccination compared to pre-trial responses an augmented reaction was noted after the subsequent vaccination. The response appeared to be more robust to the immunoadjuvant KLH than to the autologous tumour lysate.



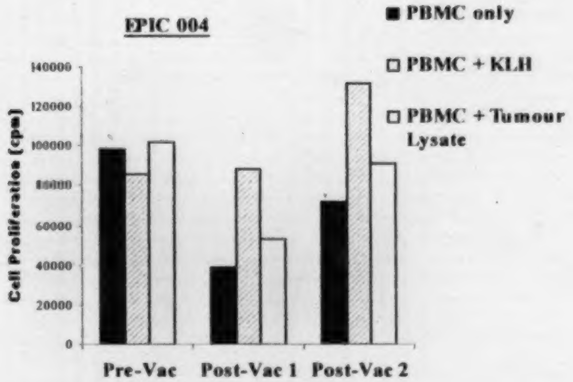


Figure 3-6 **Patient mixed lymphocyte proliferation** measured using H^3-thymidine uptake. Lymphocyte activity in response to various stimuli was compared both before and after trial vaccine injections.

We also looked at the response of these patient's mixed lymphocytes with respect to IFNγ secretion following stimulation. These results are depicted in **Figure 3-7**. Interestingly, there was no significant IFNγ response in EPIC 001 following vaccination regardless of the *in vitro* stimulant. For EPIC 004, however, there was a significant increase in IFNγ secretion following vaccinations. This response was incremental with subsequent vaccinations and was most robust for *in vitro* stimulation with KLH and tumour lysate.

Additionally we evaluated the cytotoxic ability of the patients' mixed lymphocytes against autologous tumour cells using a Cr^{51} release assay. The results are displayed in **Figure 3-8**. For EPIC 001 no significant tumour cell target killing was noted either pre or post vaccination. EPIC 004 on the other hand did demonstrate some minimal cytotoxic reactivity at higher effector to target ratios following the second vaccination.

B2. Patient Serum Evaluation

Concurrent with bloodwork drawn for safety testing serum samples were evaluated for ESR, C-reactive protein, LDH, and S100β. **Figures 3-9** to **3-12** demonstrate graphically the patients' serum levels of these markers while on protocol.

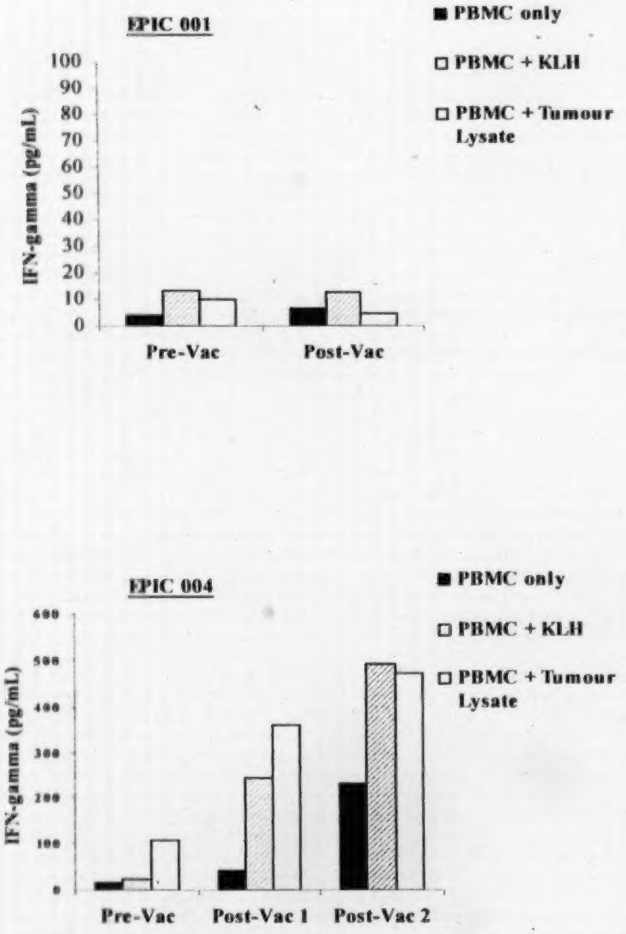
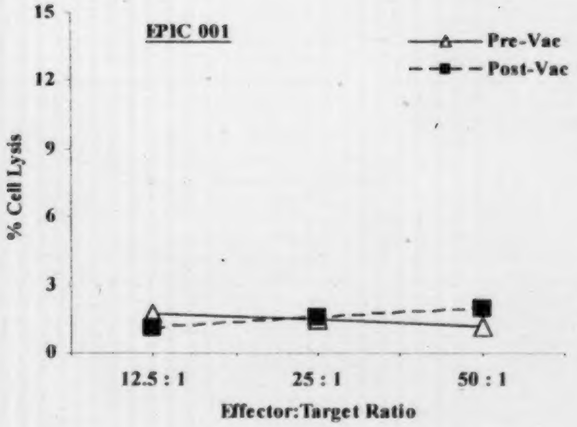


Figure 3-7 **Interferon gamma secretion by patient mixed lymphocytes** was detected in culture media using a standard ELISA kit. Lymphocyte activity in response to various stimuli was compared both before and after trial vaccine injections.



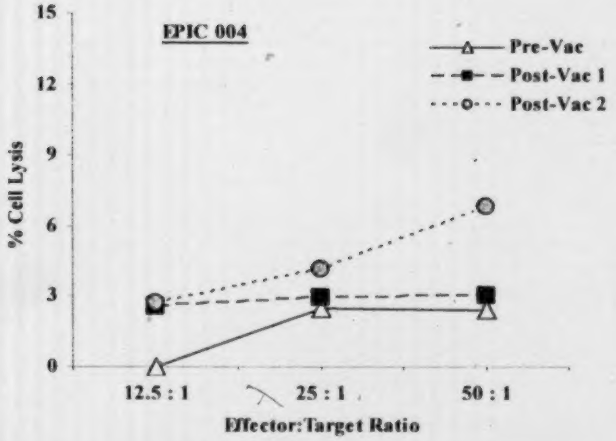


Figure 3-8 **Mixed peripheral lymphocyte cytotoxicity against autologous tumour cells**. Patients' circulating peripheral lymphocytes were mixed with autologous early explant tumour culture cells. Percent specific target tumour cell lysis was detected using a Cr^{51} release assay.

Figure 3-9. Evaluating ESR levels it was found that EPIC 001 demonstrated a progressive increase in ESR from the upper range of normal (0-10 mm/hr) to greater than 60 mm/hr just prior to succumbing to the disease. EPIC 004 had significantly elevated levels, > 50 mm/hr from the outset of the vaccination protocol. EPIC 006 demonstrated high borderline values consistently with a final value at V6 (vaccine injection 6) of 17 mm/hr. EPIC 007 had normal ESR values throughout.

Using 8.0 mg/l as the upper limit of normal for serum CRP, **Figure 3-10**, EPIC 001 was noted to have elevated levels at the beginning of the protocol which continued to escalate to values over 160 mg/L during treatment and consistent with clearly progressive clinical disease. EPIC 004 had a decline in CRP levels from above normal levels at V1 to upper normal values by V2 and throughout the protocol. Both EPIC 006 and EPIC 007 consistently were found to have CRP values well below 8.0 mg/L.

The normal serum concentration of LDH is from 100-225 U/L. Over the course of vaccinations and concurrent with disease progression EPIC 001 was observed to have marked elevation of LDH, **Figure 3-11**. All other patients had LDH levels within normal limits.

For S100β, **Figure 3-12**, only EPIC 001 demonstrated a progressive elevation of S100β levels above 0.12 ng/ml. EPIC 004 had a single spurious

Figure 3-9. Trial patient serum ESR levels vs time.

Serum ESR level data points are displayed on a 52 week on-protocol timeline. ESR levels are expressed in mm/h. The normal range for serum ESR is 0 – 10 mm/h. For patient, EPIC 001 the * indicates times where vaccination and labwork were concurrent. For all other patients the labwork was drawn just prior to the next vaccination. ** indicates time of death if that occurred during the one year trial observation period. For patient EPIC 001 during the period indicated by the bar with arrows the patient received 6 vaccinations at one week intervals.

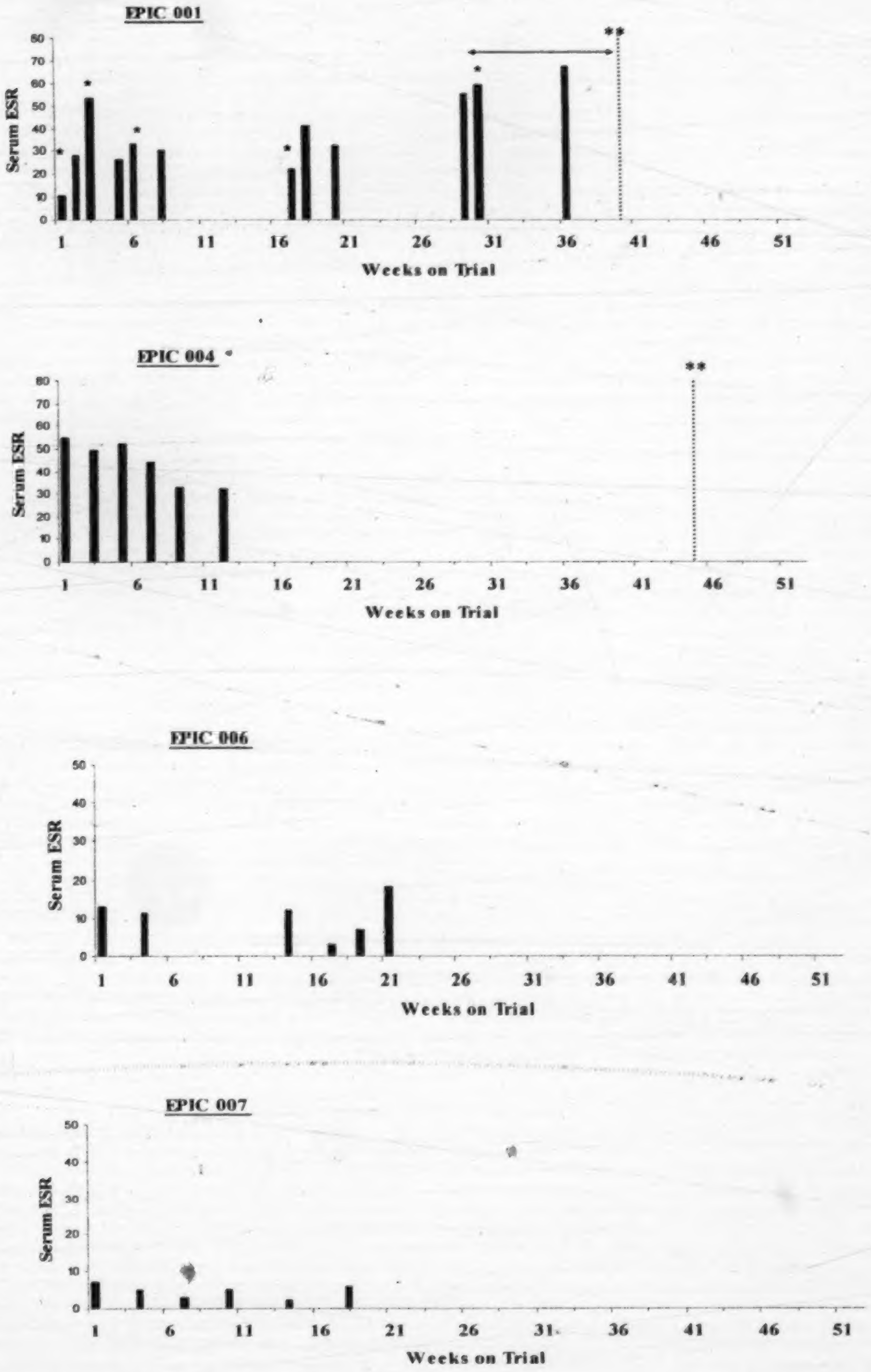

EPIC 001
Serum ESR
0 10 20 30 40 50 60 70 80
*
**
1 6 11 16 21 26 31 36 41 46 51
Weeks on Trial
EPIC 004
Serum ESR
0 10 20 30 40 50 60 70 80
**
1 6 11 16 21 26 31 36 41 46 51
Weeks on Trial
EPIC 006
Serum ESR
0 10 20 30 40 50
1 6 11 16 21 26 31 36 41 46 51
Weeks on Trial
EPIC 007
Serum ESR
0 10 20 30 40 50
1 6 11 16 21 26 31 36 41 46 51
Weeks on Trial

Figure 3-9

Figure 3-10. Trial patient C-reactive protein levels vs time.

Serum C-reactive protein data points are displayed on on a 52 week on-protocol timeline. C-reactive protein levels are expressed in mg/L. The normal range for C-reactive protein is less than 8.0 mg/L. For patient, EPIC 001 the * indicates times where vaccination and labwork were concurrent. For all other patients the labwork was drawn just prior to the next vaccination. ** indicates time of death if that occurred during the one year trial observation period. For patient EPIC 001 during the period indicated by the bar with arrows the patient received 6 vaccinations at one week intervals.

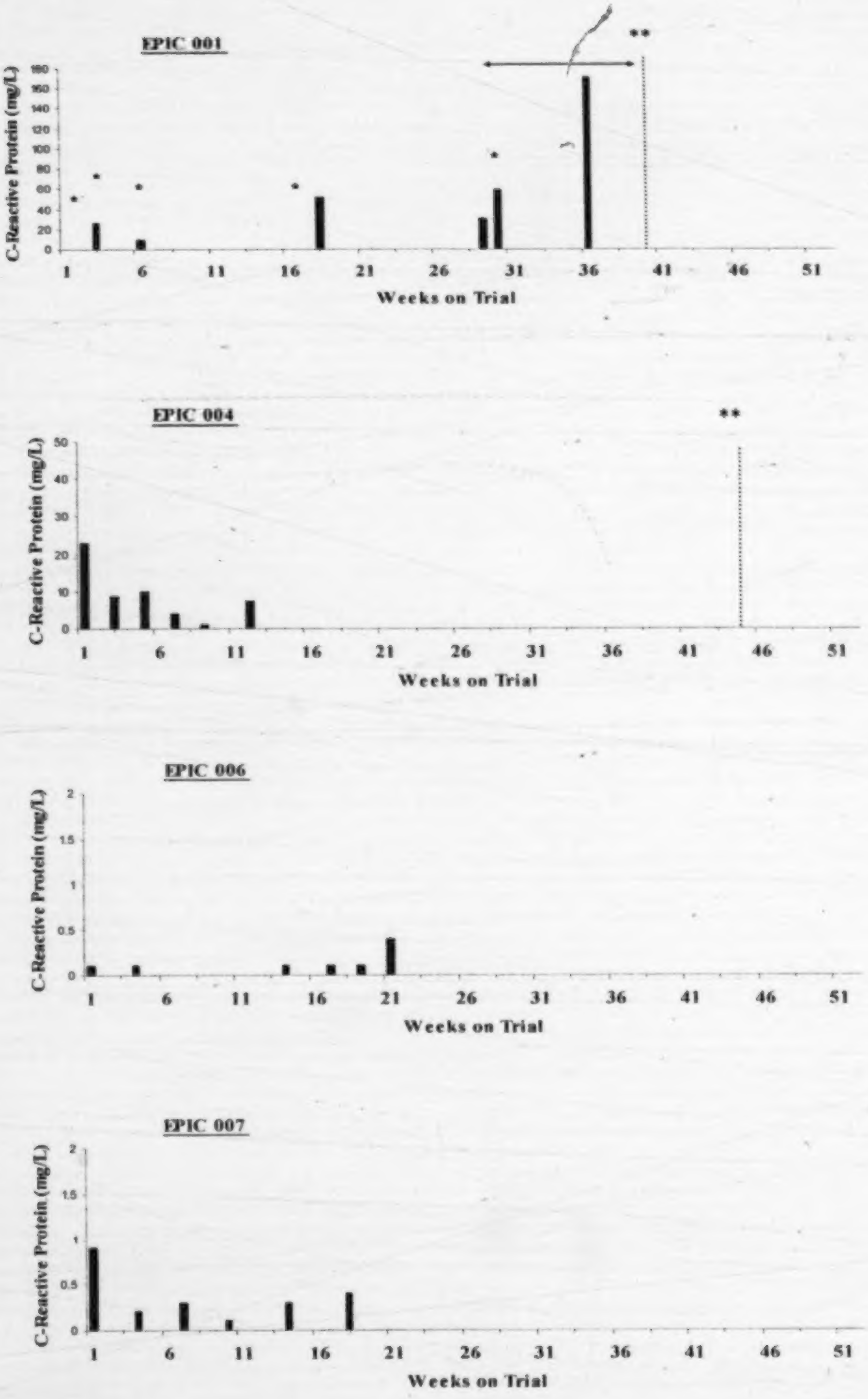

EPIC 001
C-Reactive Protein (mg/L)
180
160
140
120
100
80
60
40
20
0
*
**
Weeks on Trial
EPIC 004
50
40
30
20
10
0
**
EPIC 006
2
1.5
1
0.5
0
EPIC 007
1
6
11
16
21
26
31
36
41
46
51

Figure 3-10

Figure 3-11. **Trial patient lactate dehydrogenase levels vs time.**

Serum lactate dehydrogenase data points are displayed on on a 52 week on-protocol timeline. Lactate dehydrogenase levels are expressed in U/L. The normal range for lactate dehydrogenase is 100-225 U/L. For patient, EPIC 001 the * indicates times where vaccination and labwork were concurrent. For all other patients the labwork was drawn just prior to the next vaccination. ** indicates time of death if that occurred during the one year trial observation period. For patient EPIC 001 during the period indicated by the bar with arrows the patient received 6 vaccinations at one week intervals.

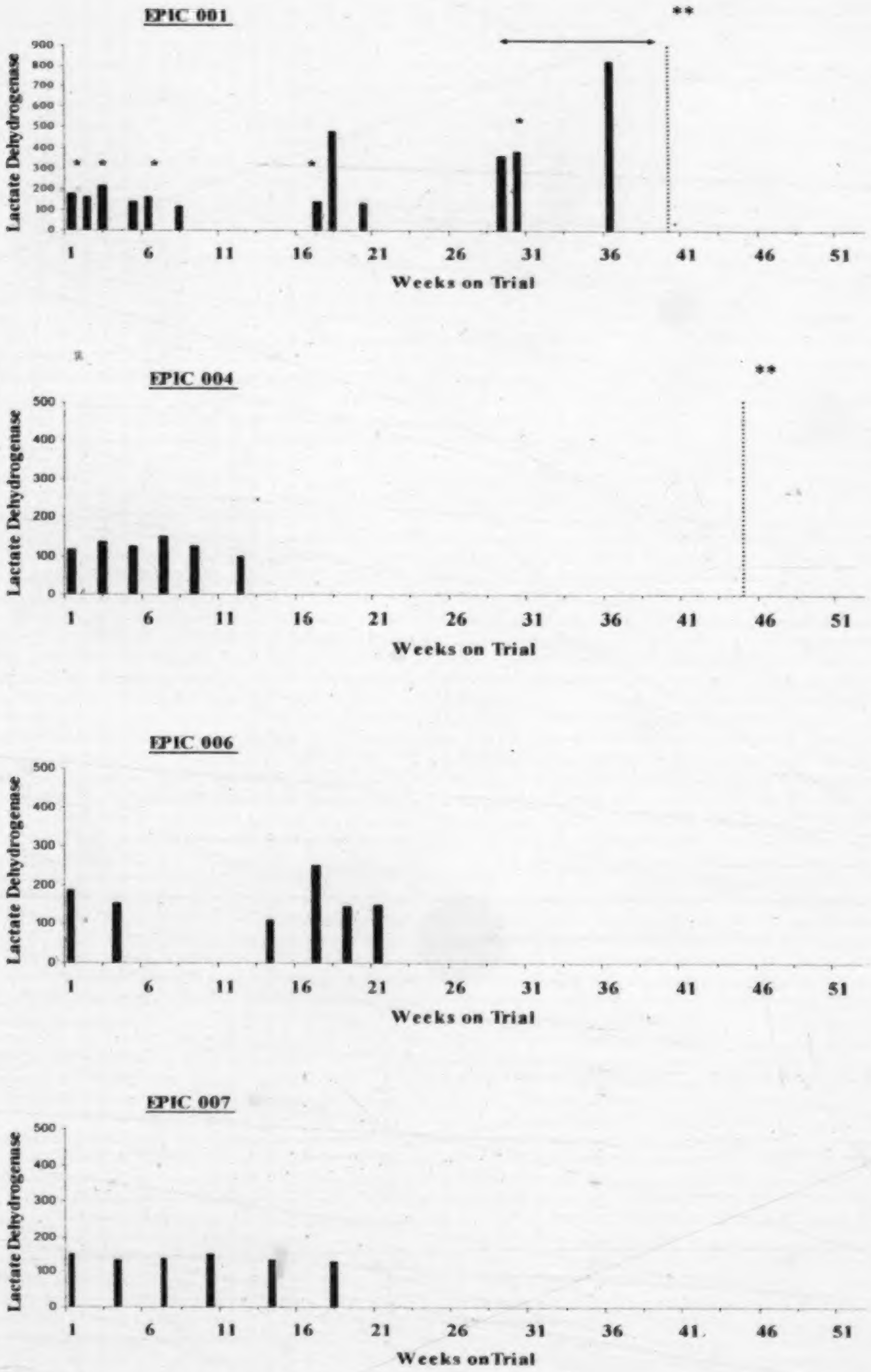

EPIC 001
**
*
*
*
*
*
Lactate Dehydrogenase
900
800
700
600
500
400
300
200
100
0
1
6
11
16
21
26
31
36
41
46
51
Weeks on Trial
EPIC 004
**
Lactate Dehydrogenase
500
400
300
200
100
0
1
6
11
16
21
26
31
36
41
46
51
Weeks on Trial
EPIC 006
Lactate Dehydrogenase
500
400
300
200
100
0
1
6
11
16
21
26
31
36
41
46
51
Weeks on Trial
EPIC 007
Lactate Dehydrogenase
500
400
300
200
100
0
1
6
11
16
21
26
31
36
41
46
51
Weeks onTrial

Figure 3-11

Figure 3-12. Trial patient serum S100-β levels vs time.

Serum S100-β data points are displayed on on a 52 week on-protocol timeline. Serum S100-β levels are expressed in ng/mL. The normal range for S100-β is < 0.10 ng/mL. For patient, EPIC 001 the * indicates times where vaccination and labwork were concurrent. For all other patients the labwork was drawn just prior to the next vaccination. ** indicates time of death if that occurred during the one year trial observation period. For patient EPIC 001 during the period indicated by the bar with arrows the patient received 6 vaccinations at one week intervals.

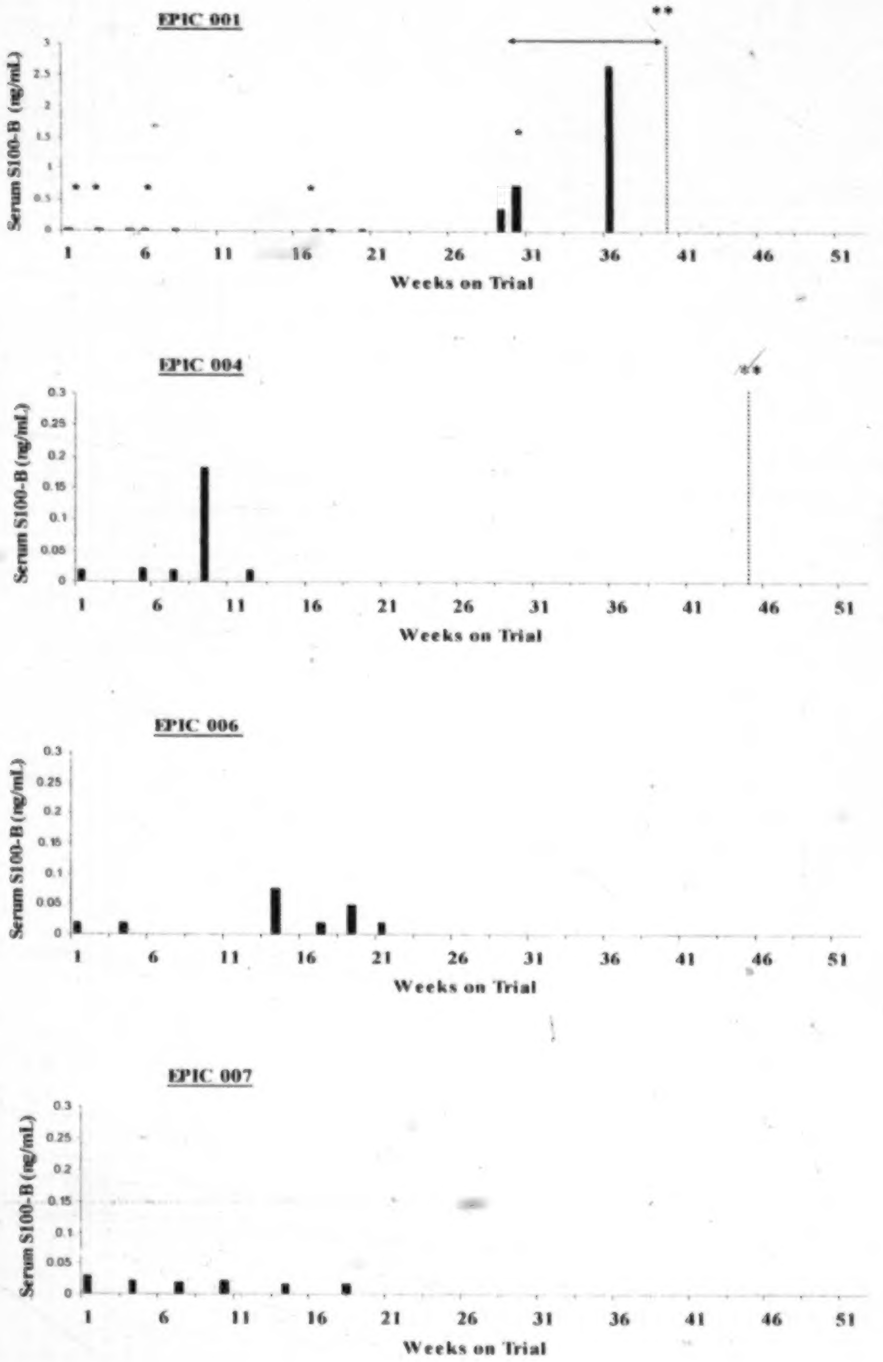

EPIC 001
**
Serum S100-B (ng/mL)
0
0.5
1
1.5
2
2.5
3
1
6
11
16
21
26
31
36
41
46
51
Weeks on Trial
EPIC 004
**
Serum S100-B (ng/mL)
0
0.05
0.1
0.15
0.2
0.25
0.3
Weeks on Trial
EPIC 006
Serum S100-B (ng/mL)
Weeks on Trial
EPIC 007
Serum S100-B (ng/mL)
Weeks on Trial

Figure 3-12

increase to 0.17 ng/ml concurrent with V4. Both EPIC 006 and EPIC 007 had normal values throughout.

The results of serum immunolglobulin levels are displayed in **Table 3-1**. Laboratory IgG levels for EPIC 001 revealed below normal titers for total IgG, IgG1, and IgG4 a the outset of vaccinations. These values remained below normal throughout the course of treatment. Interestingly, IgG2 levels declined with disease progression. Patient EPIC 004 had normal total IgG values throughout. However, above normal titers of IgG2 and IgG4 were noted early. IgG2 levels declined sharply between V5 and V6 concurrent with clinical progression of disease, but remained within normal levels. EPIC 006 was found to have below normal total IgG and IgG1 at V5 and V6. Titers were normal throughout for EPIC 007.

B3. Pathology Evaluation

We performed microhistopathology studies to evaluate the degree of immune response to the vaccination protocol at the tumour tissue level.

EPIC 001: Small fragments of intrathecal metastasis resected from the Thoracic-12 to Lumbar-1 level approximately 3 months after initiating the vaccination protocol were evaluated. The tissue demonstrated proliferating regions of tumour as well as some necrotic regions. There was significant angiogenesis with

Table 3-1. Serum Immunoglobulin Levels

Total and subtype levels of IgG immunoglobulins were measured from patient serum samples during the vaccination trial period. * Indicates values that are below normal serum levels. † Indicates IgG values that are above normal serum ranges.

EPIC 001:

	TOTAL IgG (6.94-16.18)	**IgG 1** (4.56-8.93)	**IgG 2** (1.89-5.27)	**IgG 3** (0.17-1.0)	**IgG 4** (0.07-0.74)
V1	6.41 *	3.73 *	3.11	0.35	0.02 *
V2	5.84 *	4.04 *	3.01	0.44	0.01 *
V3	5.97 *	3.39 *	2.77	0.19	0.01 *
V4	6.04 *	3.75 *	2.42	0.37	0.02 *
V5	5.09 *	3.27 *	1.72 *	0.27	0.04 *
V10	5.67 *	4.23 *	0.69 *	0.24	0.02 *

EPIC 004:

	TOTAL IgG (6.94-16.18)	**IgG 1** (4.56-8.93)	**IgG 2** (1.89-5.27)	**IgG 3** (0.17-1.0)	**IgG 4** (0.07-0.74)
V1	15.13	8.70	**7.03 †**	0.85	**1.24 †**
V2	15.67	**9.36 †**	**6.74 †**	0.89	**1.34 †**
V3	15.33	8.64	**6.71 †**	0.81	**1.52 †**
V4	16.02	8.48	**6.96 †**	0.86	**1.39 †**
V5	14.88	8.25	**6.41 †**	0.79	**1.25 †**
V6	9.85	6.19	4.34	0.48	0.69

EPIC 006:

	TOTAL IgG (6.94-16.18)	**IgG 1** (4.56-8.93)	**IgG 2** (1.89-5.27)	**IgG 3** (0.17-1.0)	**IgG 4** (0.07-0.74)
V1	7.56	4.84	2.54	0.80	0.18
V2	7.63	5.49	2.98	0.89	0.19
V3	7.62	4.98	2.64	0.78	0.18
V4	8.19	5.18	2.29	0.69	0.17
V5	5.99 *	3.70 *	2.01	0.64	0.10
V6	6.56 *	4.39 *	2.18	0.56	0.12

EPIC 007:

	TOTAL IgG (6.94-16.18)	**IgG 1** (4.56-8.93)	**IgG 2** (1.89-5.27)	**IgG 3** (0.17-1.0)	**IgG 4** (0.07-0.74)
V1	10.74	7.66	2.25	0.50	0.46
V2	10.68				
V3	10.85	7.62	2.34	0.48	0.44
V4	10.20				
V5	11.03				
V6	11.68	7.60	2.13	0.54	0.54

Table 3-1

perivascular nuclear free zones and occasional mitotic figures. **Figure 3-13** displays images of specific immunohistochemical preparations and **Table 3-2** summarizes the relative responses of the various markers studied. CD4 positive cells were significantly present within the tumour parenchyma, **Figure 3-13A**. The number and distribution of CD25 bearing cells (a marker for T-regulatory lymphocytes) paralleled that of CD4, **Figure 3-13B**. Significant infiltration of tumour was also noted for CD8 positive cells, **Figure 3-13C**. However, the number of CD69 positive cells (activated T-cell marker), **Figure 3-13D**, was consistently less than CD4 or CD8 positive cells. CD68, a macrophage marker, showed moderate numbers of infiltrating cells within the tumour itself, **Figure 3-13E**, and as expected higher numbers were seen at necrotic margins. Interestingly, strong staining of CD45RO, indicative of memory T-lymphocytes, was seen throughout, **Figure 3-13F**. Unfortunately post-mortem tissue for EPIC 001 was not available for immunohistological evaluation.

EPIC 004: A gross total resection of a right temporal lobe, 3.5 cm, metastatic lesion was performed two months after the initial vaccination. The tissue specimens demonstrated areas of gross and micro-hemorrhage as well as regions of homogeneous proliferating tumour and areas of rarefied loose reticulated tissue and necrosis. Micro areas of hemosiderin deposition were noted. Scattered CD4 positive cells were observed to be sparse within dense tumour and more prolific within the loose reticulated areas. There was no perivascular predilection noted. CD8 positive cells were not as numerous as

Patient	CD 4	CD 25	CD 8	CD69	CD45RO	CD68
EPIC 001	+++	+++	+++	++	+++	++
EPIC 001B	*	*	*	*	*	*
EPIC 004A	++	++	++	+	+	+
EPIC 004B	++	++	++	+	+	++
EPIC 006	+	+	+	Absent	+	++
EPIC 006B	**	**	**	**	**	**
EPIC 007A	Rare	+	+	+	Absent	++
EPIC 007B	+++	+++	+++	+++	Absent	+++
EPIC 007C	***	***	***	***	***	***

Table 3-2. Pathology Evaluation of Immune Cell Infiltration

Grading of positive immunohisochemical studies on trial patient paraffin embedded tissue samples. + indicates occasional positive cell, ++ indicates several positive cells, and +++ indicates prolific presence of positive cells. EPIC 001 tissue was from an intrathecal metastasis occurring at 3 months following initiation of the trial. EPIC 004A tissue was from a right temporal lobe metastatic lesion occurring 2 months after initial vaccination. EPIC 004B tissue was from a post-mortem, left inferior parietal metastatic lesion 10.5 months from the initial vaccination. EPIC 006 tissue was from a left frontal lobe intracranial metastasis prior to starting vaccinations. EPIC 007A tissue was from a left ankle lesion prior to vaccination. EPIC 007B tissue was taken form a left ankle recurrent lesion 16 months after the initial vaccination.

* EPIC 001 post-mortem tissue unavailable for immunohistochemistry.

** EPIC 006 post-mortem tissue unavailable due to denied consent.

*** EPIC 007 patient continues to survive therefore post-mortem tissue unavailable.

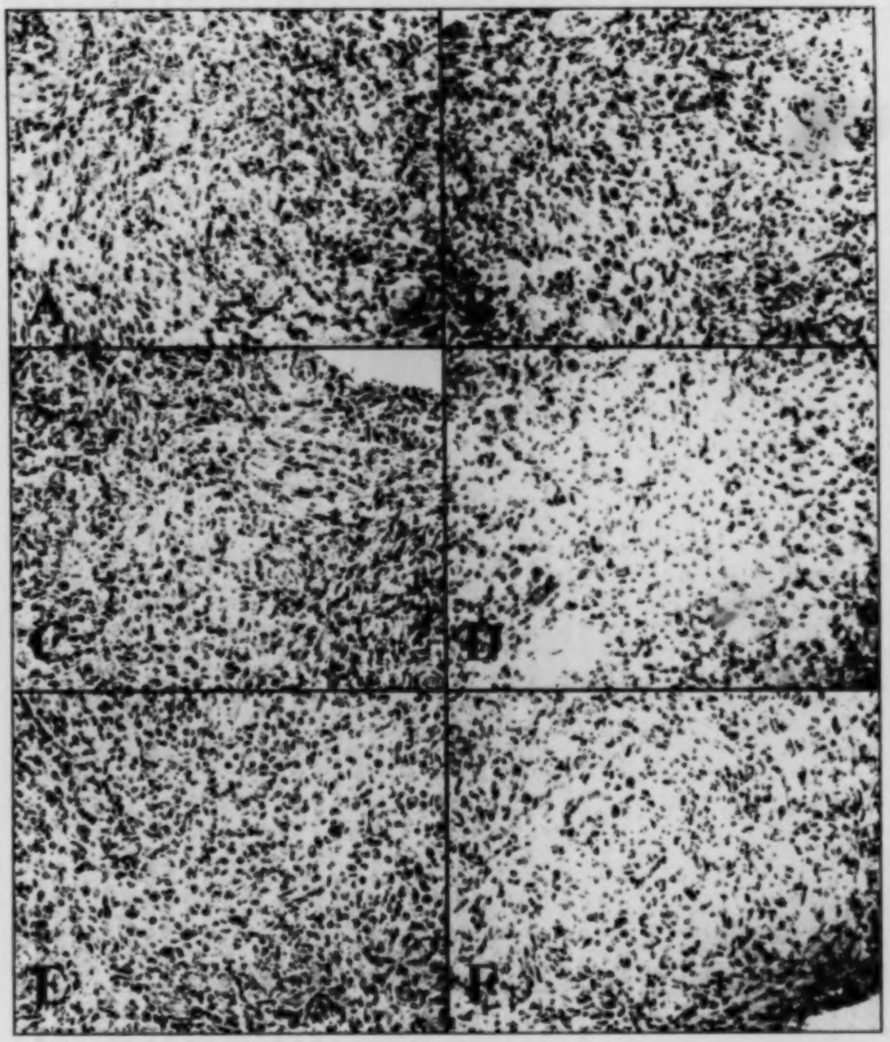


Figure 3-13 Immunohistochemistry EPIC 001

Immunohistochemical studies performed on paraffin embedded tissue from EPIC 001- an intrathecal metastatic lesion identified and resected at 3 months following initial vaccination. **A** – anti CD4, **B** – anti CD 25, **C** – anti CD 8, **D** – anti CD 69, **E** – anti CD 68, **F** – anti CD 45RO. All images at 20X magnification.

CD4s but parallel in distribution. Rare, but focal clusters of CD25 positive cells were observed. Very rare CD69 positive cells were seen. Very rare isolated CD83 positive cells (DC marker) were seen within tumour parenchyma. Also, rare macrophages, CD68 positive cells, were noted in tumour parenchyma but prevelant in necrotic/fibrillary areas.

Post-mortem tissue taken from a left parietal lesion at 10.5 months following the first vaccination was also evaluated. **Figure 3-14** portrays the immunohistochemical findings. **Table 3-2** displays the relative immune cell response. H&E preparation revealed tumour with areas of micro-hemorrhage. At the tumour-brain parenchyma interface, reactive gliosis and fibrotic change was seen. Some areas were consistent with older micro-hemorrhage and macrophages laden with hemosiderin were present. There were some focal areas of intense CD4 staining within clearly fresh hematoma. As seen in **Figure 3-14A** there was only sparse infiltration within the tumour parenchyma. There was a predilection noted for the tumour margin and no CD4 positive cells were seen in the surrounding normal brain parenchyma. The distribution of CD25 positive cells was parallel to that of CD4s – **Figure 3-14B**. CD8 positive cells were similarly sparse in tumour, **Figure 3-14C**, absent from normal parenchyma, and focally distributed to regions of micro and gross hemorrhage. Only the rare and isolated CD69 positive cell was noted. As seen in **Figure 3-14E** macrophages, CD68 positive, were found prominent and concentrated at the tumour-brain parenchyma interface within the gliotic reactive region. They were also distributed as expected

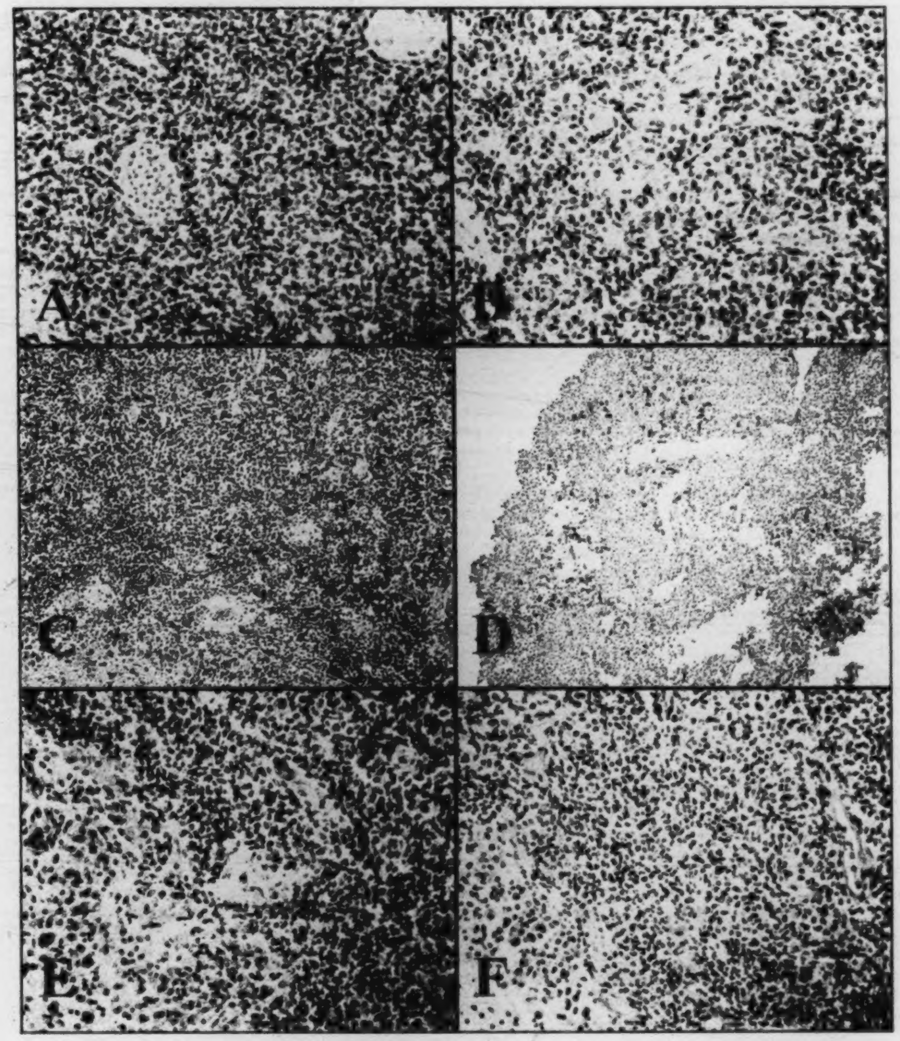


Figure 3-14 Immunohistochemistry EPIC 004

Immunohistochemical studies performed on paraffin embedded tissue from EPIC 004; post-mortem left inferior temporal metastatic intracranial lesion at 10.5 months following initial vaccination. **A** – anti CD4, **B** – anti CD 25, **C** – anti CD 8, **D** – anti MIB1, **E** – anti CD 68, **F** – anti CD 45RO. Images A, B, E, F at 20X magnification. Images C, and D at 10X magnification.

to areas of hemorrhage. **Figure 3-14F** shows the sparse CD45RO reactivity indicative of memory T-lymphocytes. Finally, **Figure 3-14D** shows the significant MIB1 (mindbomb homologue 1) positivity associated with the aggressive proliferating nature of this intracranial metastatic lesion.

EPIC 006: A gross total resection of a left frontal brain metastasis was performed prior to the patient being enrolled in the vaccination trial. The pathology evaluation of immune cell response is summarized in **Table 3-2**, and images of immunohistochemical evaluation are displayed in **Figure 3-15**. On H&E preparation of the resected tissue revealed uniform tumour tissue and no normal adjacent brain parenchyma. No areas of necrosis or rarefied fibrosis were noted. Angiogenesis, micro-hemorrhage and small hemosiderin deposits were noted, **Figure 3-15E**. As observed in **Figure 3-15A**, few CD4 positive cells were present. Some more focal areas of positivity were noted but those were in areas consistent with micro-hemorrhage or surgical trauma. Similarly, only rare truly positive CD8 cells were seen and those were in proximity to regions where hemosiderin was deposited. No CD69 positive cells were seen. CD68 positive cells were noted to have a predilection for areas where hemosiderin was deposited within the tumour, **Figure 3-15D**, or regions of micro-hemorrhage. Diffuse positive staining for CD45RO was noted throughout the tissue samples, **Figure 3-15F**. Unfortunately, access to post-mortem tissue samples was not permitted for comparisons to be made.

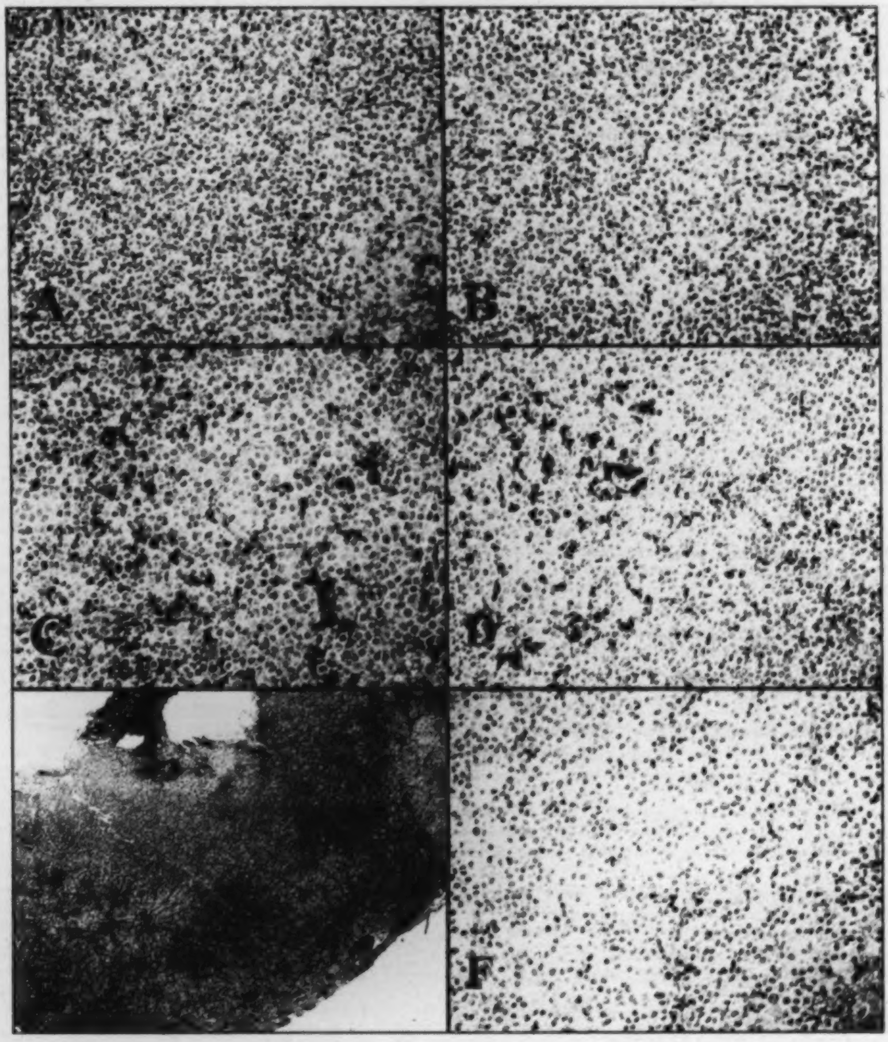


Figure 3-15 Immunohistochemistry EPIC 006

Immunohistochemical studies performed on paraffin embedded tissue from EPIC 006- a left frontal lobe intracranial metastatic lesion resected prior to the initial vaccination. **A** – anti CD4, **B** – anti CD 25, **C** – anti CD 8, **D** – anti CD 68, **E** – H & E, **F** – anti CD 45RO. Image E at 2X magnification. All other images at 20X magnification.

EPIC 007: For this patient tumour tissue samples from both prior to starting the vaccination protocol and from four months following completion of the trial were available and compared. The relative immune cell response is summarized in **Table 3-2**, and representative immunohistochemical preparations are presented in **Figure 3-16**. Images **3-16 A,C,E,G** and **I** are derived from tumour resection of a left ankle lesion prior to vaccination. Images **3-16 B,D,F,H** and **J** are from a resected tumour recurrence at the left ankle 4 months after completing the 1 year vaccination protocol. We found that for all markers examined there was a significant proliferation of immune cells following the patient's vaccination treatments.

B4. Patient Survival

In this study we were also interested in whether or not patient survival was modified by undergoing the autologous vaccination protocol. As noted in the timeline figures for each patient, **Figures 3-2** to **3-5** all patients had progression of disease while on the vaccination protocol. **Tables 3-3 and 3-4** summarize the extent of treatment and survival times for each patient. The survival of EPIC 001, 004, and 006, all with AJCC stage IV disease include CNS involvement was 14, 8, and 29 months respectively from the time of diagnosis with brain lesions. The mean survival of this small group with cerebral melanoma metastases was therefore 17 months. It is also of particular interest that EPIC 007 progressed

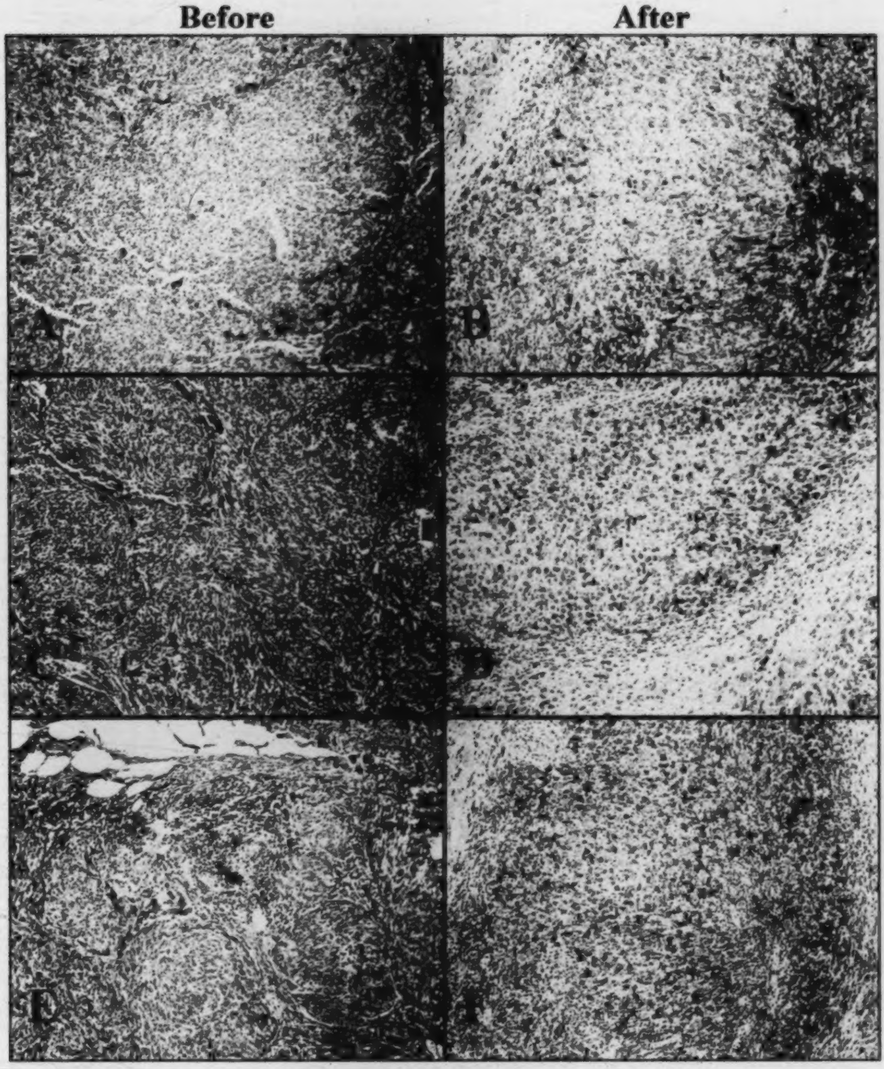


Figure 3-16 Immunohistochemistry EPIC 007

Immunohistochemical studies performed on paraffin embedded tissue from EPIC 007. A, C, E, G, and I are images of preparations of tissue resected from a left ankle lesion prior to vaccination. B, D, F, H, and J are images of tissue taken from a left ankle recurrent lesion 4 months after completing the 1 year vaccination protocol. **A&B** – anti CD4, **C&D** – anti CD 8, **E&F** – anti CD 68, **G&H** – anti CD 25, **I&J** – anti CD 69. All images at 10X magnification.

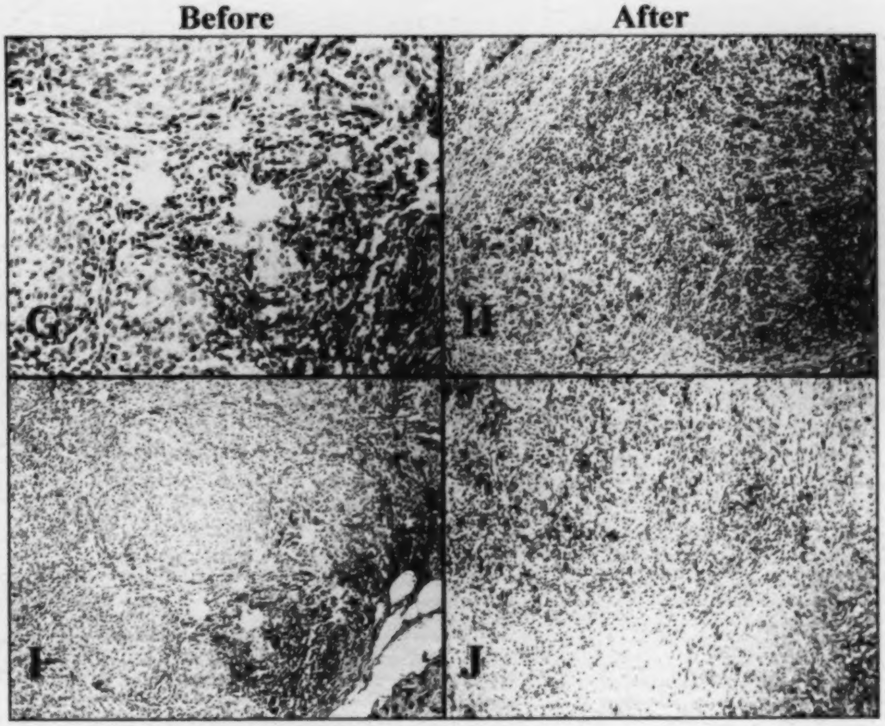

Before
After
G
H
I
J

Figure 3-16 continued

Table 3-3

Trial No.	Tumour Path.	Age	Gender	KPS	Pre-vac. Treatment	Vaccinations	Dates on Trial
EPIC 001	AJCC IV	52	F	90	• Surgery x4 • Isolated limb perfusion • Chemotherapy x2 • γ knife radio surgery	11	17/09/03 27/06/04
EPIC 004	AJCC IV	17	F	100	• Surgery x3 • Chemotherapy • Radiation	6	1/09/04 12/07/05
EPIC 006	AJCC IV	48	F	100	• Surgery x2 • Isolated limb perfusion x2	6	25/11/04 25/11/05
EPIC 007	AJCC III	65	F	100	• Surgery x4 • Radiation x2	6	14/07/04 14/07/05

Table 3-4

Trial No.	Initial Diagnosis	Tumour Status prior to V1	Concurrent Trial Interventions	Survival from initial diagnosis	Survival time with brain mets	Survival from V1
EPIC 001	1982: left leg solitary lesion	Inguinal nodes, mediastinal nodes, left parietal brain metastasis	• surgery for spinal intrathecal met. • whole brain and stereotactic radiation for brain metastasis and progresion	22 years	14 months	9 months
EPIC 004	Nov. 2002: left scapular nevus	Primary scar recurrence, left axillary nodes, left iliac crest	• surgery for brain metastasis • whole brain radiation and stereotactic radiation for brain metastases and progression	45 months	8 months	10 months
EPIC 006	June 1997:left cheek solitary lesion	Primary site recurrence, cervical nodes, left lung apex, left frontal brain metastases	• stereotactic radiation to single brain metastasis	7.5 years	29 months	17 months
EPIC 007	Nov. 2000: left ankle solitary lesion	Left inguinal nodes, left leg lesions x4	• surgical resection of metastases to right arm and left chest wall	6 years +, Patient alive	No CNS metastases	24 months + Patient alive

from AJCC stage III to stage IV during the vaccination protocol but has continued to survive beyond 32 months from the first vaccination.

Discussion:

The purpose of this phase I trial was to determine the safety and efficacy of utilizing an adoptive immunotherapy strategy consisting of autologous CD14+ monocyte derived dendritic cells primed with an autologous tumour peptide extract in combination with immunoadjuvant therapy to promote anti-tumour responses for the treatment of medically refractory melanomas.

Adoptive immunotherapy involves the administration of immune effector cells that have been collected and expanded or manipulated *ex vivo*, before being returned to the patient/subject. This strategy attempts to circumvent the immune regulatory and generally suppressive influence exerted by tumours. As noted previously it has been demonstrated that the degree of lymphocyte infiltration into melanomas is an important independent prognostic factor for survival[11]. An early adoptive strategy against melanoma sought to use tumour infiltrating lymphocytes (TILs).

TILs can be grown from a tumour cell suspension in IL-2 containing media[35, 36]. The largest applications of this strategy for metastatic melanoma involved the treatment of 86 and 134 patients with TILs and high dose IL-2[37, 38]. It was found that about one third of patients had only partial and short lived responses. Poor clinical outcomes with this treatment continue despite the objective finding that radiolabelled IL2 boosted TILs localized to tumour sites *in vivo*[39]. Another strategy utilizes circulating cytotoxic T-lymphocytes which are isolated and stimulated *ex vivo*. These lymphokine activated killer T-cells (LAKs) are returned to patients. Studies however, comparing treatment with systemic IL-2 alone with IL-2 and LAKs determined no clinical differences and so treatment involving LAKs is no longer utilized[40, 41].

Vaccinations using whole tumour have been used in an attempt to present a diverse antigen profile to the immune system with the advantage of not having to know or isolate the specific antigen(s) that may be responsible for tumour cytotoxicity. An example of this strategy was employed by Berd et. al. who demonstrated safety and efficacy using irradiated autologous tumour cell vaccination with BCG and chemotherapy[42]. However, only 11% of patients had a response in AJCC stage IV disease. A leading criticism of this strategy is that the immune system is simply being exposed to antigens to which it is already tolerant or anergic. It is significant that a recent randomized phase III trial of an allogeneic whole cell vaccine, CancerVax®, was stopped early by the data and safety monitoring board after treatment of 496 patients revealed a lack of efficacy compared to controls. Attempts have been made to make autologous cells more immunogenic by transfecting the tumour cells such that they come to express immune activating molecules such as GM-CSF and B7-1[43, 44]. The safety concerns over vaccination treatment with virally transfected cells and the issue of significant delays to treatment due to manufacturing have made this approach impractical.

The observation that tumours from different patients share multiple tumour specific antigens has made the use of peptide vaccination the focus of many clinical trials. Some examples of these identified tumour antigens include MAGE1[45], gp100[46], tyrosinase[47], and MART1[48]. Taking advantage of these identified antigens it has been possible to evaluate melanoma patients for specific circulating T-lymphocytes[49]. A meta-analysis of peptide vaccination for stage IV melanoma after resection of metastases[50] with MAGE 3[51], MART 1[52], gp100/ tyrosinase[53], gp100/tyrosinase/MART 1[54], or gp100/tyrosinase/MART 1/CTLA4 antibody[55], have suggested both clinical safety and efficacy. The ongoing criticism of the defined peptide vaccination approach is that it fails to address the breadth of antigen epitopes amongst the heterogenous are clones that make up a tumour. Further, most of these peptides MHC restricted which prevents them from being universally applicable. Finally, and perhaps most importantly many of the peptides used likely represent epitopes for which patients are already tolerized or anergic.

Dendritic cells are professional antigen presenting cells that are now recognized as the most powerful regulators of the immune system[23]. They possess the unique ability to activate and modulate both the innate and acquired immune systems. While immature DCs efficiently capture and process foreign (including tumour) antigens, mature DCs specialize in antigen presentation to naïve CD4+ and CD8+ T-lymphocytes and are effective stimulators of natural killer cells (NK) and B-lymphocytes. Expression of MHC class II, co-stimulatory molecules (CD80, CD83, CD86, CD40), adhesion molecules (CD54, CD58), and cytokines/chemokines (IL-2, IL-12, IL-15) by mature DCs results in activation of cellular cytotoxic response against target cells[56]

In this study we have used for the first time an entirely autologous system consisting of "naturally processed tumour peptides" and mature autologous DCs. We selected the use of naturally processes tumour peptides to pulse dendritic cells because of the elegant *in vitro* studies performed by Protti et.al. [26, 33]. DCs primed with naturally processed tumour peptides were able to induce strong and specific cytotoxic T-cell responses against target tumour cells. Of particular importance was the finding that the CTLs activated by this method were able to lyse both autologous and allogeneic target tumour cells but not COS cells which had been transfected fo express various well recognized melanoma specific antigens. The clear advantage of this system for us of using this naturally processed peptide strategy is that it provides DCs the full array of potentially immunogenic epitopes for each individual tumour and it includes epitopes that may not be excluded by HLA restriction.

In contrast to the method of antigen pulsing we have chosen, the majority of clinical DC trials for melanoma patients has utilized autologous DCs primed with melanoma specific antigens[57-59]. Once again we reiterate the main criticism of this approach remains failure of clinical application based on the limitation of the range of epitopes, restriction by HLA typing, and the potential failure of some malignant cells to express the selected antigen. As an example, Schuler-Thurner et. al. [58], using MAGE 3 peptide, though able to observe induction of IFNγ producing CD8+ T-cells *in vitro*, were unable to demonstrate either a significant clinical response, or MAGE 3 specific cytotoxic T-lymphocyte (CTL) enzyme linked immunosorbent spot (ELISPOT) reactivity in fresh patient blood samples.

Two important studies have looked at the use of autologous tumour lysate to prime DCs for antigen presentation. The landmark study by Nestle et.al. looked at patients vaccinated with peptide versus tumour lysate pulsed DCs and found that 2 of 4 "lysate primed" patients had a clinical response and 4 of 4 demonstrated delayed type hypersensitivity (DTH) reaction to the adjuvant keyhole lympet hemocyanin (KLH)[28]. Recently, Hersey et. al. compared 19 patients treated with autologous DCs and tumour lysates with 14 patients treated with DCs pulsed with melanoma specific peptides[60]. These authors report 4 of 19 patients showed partial response in the lysate treated cohort compared to no responses in the melanoma specific peptide group. This suggests that the use of autologous tumour lysate has an apparent clinical advantage over treatment with specific peptide pulsed DCs.

To our knowledge the combination of a dendritic cell vaccine in combination with a comprehensive immunoadjuvant protocol to combat both systemic and tumour specific immunosuppressive effects has not previously been investigated. As indicated in the introduction, melanomas have evolved complex methods of avoiding immune recognition and suppressing immune mediated cytotoxicity. We used GM-CSF due to its ability to promote recruitment of CD34+ progenitor DCs, and stimulation of DC proliferation, maturation, and expression of: CD40, TNFα, MHC class II, and CD86 co-stimulatory molecules, all of which are pivotal in generating effector and memory T-lymphocyte responses[43, 61-65]. Interferon is included in our protocol because of its ability to activate innate immune cells, reduce tumour cell PGE_2 secretion, antagonize malignant cell TGFβ function, and enhance the apoptotic potential of tumour cells[66-71]. Il-2 as an adjuvant for melanoma treatment is well established and is part of many well established therapeutic protocols. IL-2 is a T-cell growth factor and is able to reverse IL-10, superantigen, and CD4+CD25+ T-regulatory cell induced effector T-cell anergy[72-74]. Celecoxib is a COX-2 inhibitor and is utilized in this protocol because of its recognized ability to reduce malignant cell proliferation and invasion, tumour cell secretion of PGE_2 (a potent APC and T-cell suppressant) and antagonize both vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) induced angiogenesis[75-78]. Retinoic acid was also included due to its support of tumour cell differentiation, decrease in tumour cell proliferation and metastatic potential, down regulation of matrix metalloproteinases (MMPs), decreased insulin-like growth factor binding protein 2, and increased apoptotic sensitivity of melanoma cells[79-82].

The primary endpoint of this trial was to establish the safety of this combined DC and immunoadjuvant treatment for medically refractory melanoma. The vaccination and supportive adjuvants were well tolerated by all patients. The only symptoms observed were mild fever and flu-like feeling for 24-48 hours after vaccination. Specifically, no cutaneous, CNS, renal, or thyroid reactions were observed. Only one patient was noted to have a spurious, self-limited liver enzyme elevation at mid protocol. We suggest that continuation of investigations using this protocol is reasonable from a safety perspective.

We were also interested in whether this autologous DC protocol would induce a cytotoxic immune response in patients. In the past other studies have relied on the evaluation of delayed type hypersensitivity (DTH) reactions to molecules such as KLH or bacillus Calmette-Guerin (BCG) to establish antigen specific immunity. Though a positive DTH finding with injection site infiltration of CD8+ T-cells and CD45RO cells is reassuring, it is not consistent, and when present is not correlated with clinical efficacy[28]. As a further example, peripheral subcutaneous injection of pure MAGE 3 peptide failed to induce a DTH reaction after patients had been vaccinated with MAGE 3 pulsed autologous DCs[58]. We did not evaluate the patients in our study therefore using a DTH assay.

We chose instead to evaluate the first two patients for evidence of circulating lymphocyte ability to proliferate, secrete IFNγ, and kill autologous tumour cells *in vitro*. One patient EPIC 001, though demonstrating some PBMC proliferation with KLH or tumour peptide after vaccination, the level was not above control levels. Additionally, this patient's PBMCs did not show any IFNγ secretion and they were not able to kill autologous tumour target cells. It is interesting that patient EPIC 001 had the shortest survival time. EPIC 004 on the other hand did demonstrate and enhanced lymphocyte proliferation compared to controls, though the response was greater for KLH stimulation than for tumour pepide extract. The peripheral mixed lymphocytes also showed both significant ability to secrete IFNγ – a hallmark of activated cytotoxic T-cell activation, as well as some ability to kill autologous tumour cells. Once again however, though the finding of IFNγ secretion is of interest, its specificity in terms of immune response is not well established. For example, Rosenberg et. al. in a melanoma vaccination trial found that only 2 of 8 patients had evidence of an immune response with an IFNγ assay, despite observing objective clinical responses in 42% of the 39 trial patients[83].

We were also interested in evaluating whether or not certain readily available serum markers were able to correlate with either clear immune response to vaccination or with disease progression. No clear conclusions can be made from this study because of the small sample size. We did observe however that the two patients with the shortest survival time had early elevated levels of both

ESR and CRP. Both ESR and CRP are non-specific serum reactive markers. Of late, CRP has been investigated more extensively since it was recognized to be synthesized by hepatocytes in response to various cytokines, especially IL-6[84]. IL-6 is secreted by melanoma cells and therefore CRP can be utilized as a surrogate marker for melanoma tumour burden[85]. Furthermore, elevations of CRP have been correlated with disease burden and stage progression[86]. Lactate dehydrogenase (LDH) levels have been demonstrated to be the best predictive indicator of diminished survival in melanoma patients[87]. In EPIC 001 frequent serum sampling provided a clear progressive increase in LDH levels that corresponded with disease progression. S100β is an intracellular protein that is a good immunohistochemical marker for melanoma cells. Measurement of serum S100β has been used as a marker for both staging and evaluation of disease progression[88]. Though, the main criticism of this assay is that serum testing only reflects necrosis of melanoma cells and resultant release of specifically intracellular proteins our findings of elevated levels above 0.12μg/L correlated with clear clinical progression and shortened survival are consistent with Ghanem et.al.[89].

Another important role of DCs in regulating immune responses is activation of humoural immune activity. In brief, B-lymphocytes generate immunoglobulins, specific to antigen, which ultimately opsonize a pathogen and activate complement cascade. The evaluation of circulating immunoglobulins has been used to evaluate successful vaccination. Anttila et. al. demonstrated that for *streptococcus pneumoniae* vaccination in adults, responders had elevations of total IgG, IgG1, and IgG2 titers as well as increased opsonization activity against pneumococcal capsular protein[90]. The dominating immunoglobulin subtype in adults and total IgG concentration was the most important factor in opsonization activity. Kirkwood et. al. used immunoglogulin evaluation in a phase I/II vaccine study of melanoma patients and found that IgG and IgM titer responses correlated with a trend to increased survival[71]. Also Hsueh et.al. measured specific peptide IgG and IgM responses in evaluating a progression polyvalent melanoma vaccine (CancerVax), and reported increases in IgG titer was associated with survival[91]. In our current study, the patient with the shortest survival had the lowest total IgG concentrations throughout the trial and declining IgG2 levels with end stage progression. The patients with the longest survival had the most consistent and normal IgG titers. Only one patient demonstrated a robust elevation of IgG2 and IgG4. Interestingly it was noted that with rapid disease progression IgG levels were noted to to decline.

In addition to our interest in following serum indicators of immune responsiveness and disease progression we were keen to evaluate immune responses at the tumour tissue level. In patient EPIC 006 tissue samples from before vaccination did demonstrate some immune cell infiltration. It is noteworthy that CD25+ cells indicative of regulatory T-cells were present. It is also of interest that memory cells, CD45RO+, were evident. The presence of immune cells infiltrating this "untreated" intracranial lesion was likely due to the nature of the tumour itself which had evidence of microhemorrhage and hemosiderin deposition. In other patients with CNS involvement and following vaccination treatment a more robust immune cell infiltration was present. However of equal significance is the observation that though CD8+ cells are prominent, substantially fewer in number were CD69+, indicative of activated CTLs. Also of importance is that almost equal numbers of CD4+ and CD25 positive cells are present suggesting a substantial presence and influence of T-regulatory cells. It is unclear at this time whether the presence of T-regulatory cells is consistent with the natural progression of the disease or whether this finding suggests a population of T-cells that were cross-primed by the DC vaccine. In the patient with peripheral tumour burden (EPIC 007) and the patient with the longest survival, a substantial immune cell response within tissue is observed comparing pre-vaccination with post-vaccination specimens. In this case there is a much more robust CD69+ lymphocyte presence. Yet it is again noteworthy that the munber of CD25+ cells is augmented. As stated earlier, melanoma cells are known to secrete IL-10, TGFβ, and VEGF all of which may still tip the balance away from a Th1 response despite the aggressive use of immunoadjuvants in this study. Measurement of serum IL-12 and IL-10 would be one way of monitoring this profile in the future.

Ultimately, we were interested in any impact the autologous DC vaccination would have on patient survival. We observed that all three patients that harboured intracranial metastases survived beyond the expected natural history of the disease. Median survival was 17 months (range 8-29) from the time of CNS involvement compared to the median survival of approximately 3 months reported by Sampson et.al. for 702 patients[5]. Furthermore, one patient with initial stage III disease who progressed to stage IV during the protocol continues to survive at 2 years after completion of the 1 year vaccination protocol.

At this interim report, the use of a vaccine comprised of autologous CD14+ monocyte derived dendritic cells primed *ex vivo* with autologous tumour naturally processed peptides, and supplemented with immunoadjuvant therapy is well tolerated by patients. In this study no serum marker as yet has shown clear superiority in detecting immune response or disease progression. Pathology investigations have demonstrated immune cell infiltration into tumour parenchyma following vaccination. Finally, a trend was noted toward improved survival using our protocol compared to the natural history of the disease especially for patients with melanoma affecting the CNS.

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CHAPTER 4

Evaluation of the role the Cation Independent Mannose 6 Phosphate receptor may play in avoidance of cytotoxic T-cell induced apoptosis by glioma and melanoma cell lines

Introduction:

The mannose 6-phosphate receptors (M6PRs) play a pivotal role in transport of cellular proteins and thereby in the control of cellular growth, function and viability. Two receptors have been identified: the cation–dependant mannose 6-phosphate receptor (CD-M6PR) and the cation-independent mannose 6-phosphate receptor (CI-M6PR). They are the only members of the p-type lectin family[1]. The main function of these receptors is regulation of the trafficking of newly synthesized acid hydrolases which express the M6P moiety to lysosomes [2]. In fact intracellular binding and transport appears to be the sole function of the 46 kDa CD-M6PR [3]. The CI-M6PR, however, is increasingly recognized to be a multifunctional receptor whose function is essential for normal cellular activity[4, 5].

The CI-M6PR is a 275 kDa monomeric receptor with multiple independent binding sites. The cDNA sequence for the human receptor gene consists of 9090 base pairs. The receptor protein consists of a large 2264 amino acid extracellular domain consisting of 15 contiguous repeats each of about 150 base pairs, a short 23 amino acid transmembrane region and a 164 residue cytoplasmic domain[6] – **Figure 4-1**. Though the CI-M6PR shares the function of intracellular transport of hydrolases from the trans golgi network to lysosomes the 15 repeat segments of the amino terminal end of the protein allow for binding of a number of other molecules. In 1988 the human type II insulin-like growth factor receptor gene was mapped to chromosome 6 and the sequencing identified that the receptor was in fact the same as the previously identified receptor for M6P[7]. The role of the receptor is to bind insulin-like growth factor II (IGF-

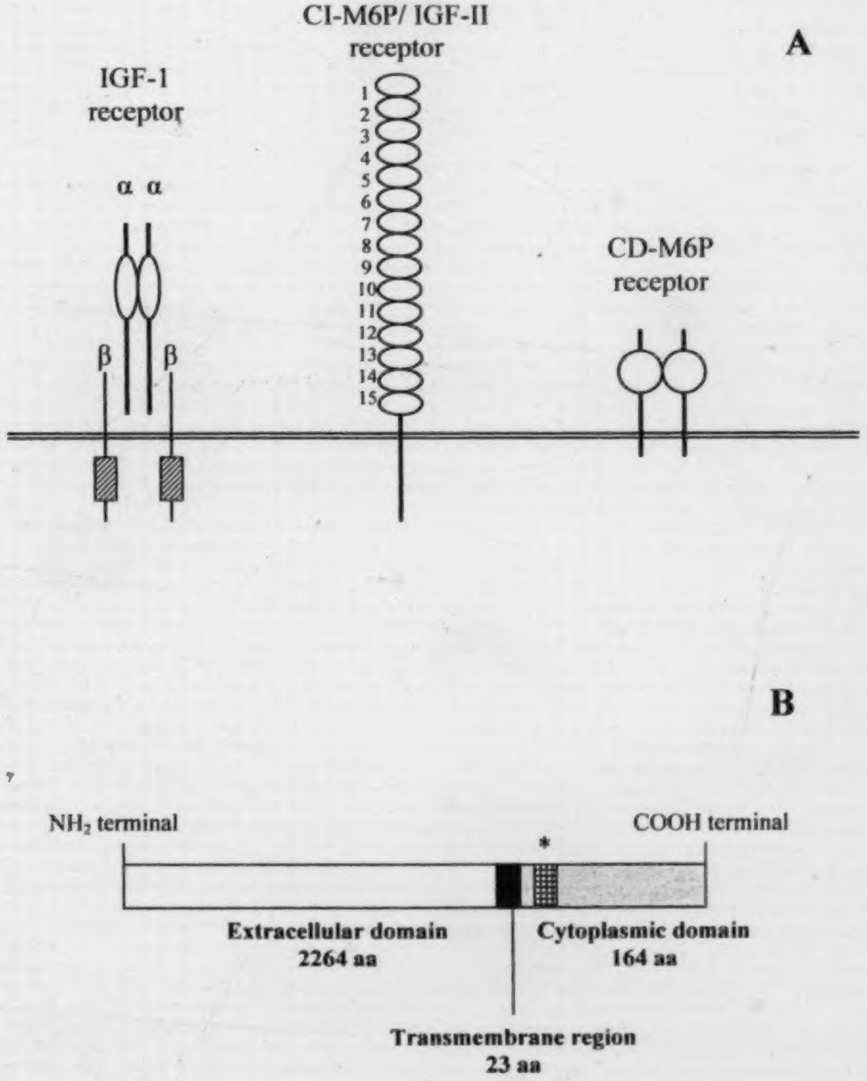

CI-M6P/ IGF-II receptor
A
IGF-1 receptor
α α
β
β
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
CD-M6P receptor
B
NH₂ terminal
COOH terminal
*
Extracellular domain 2264 aa
Cytoplasmic domain 164 aa
Transmembrane region 23 aa

Figure 4-1 **A** is a schematic representation of the CD- and CI-, M6P receptors compared to the IGF-1 receptor. **B** is a schematic of the human CI-M6PR cDNA. * This region, 24-29 base pairs 3' to the transmembrane region, is responsible for maintaining trafficking capability of the receptor.

II) at the cell membrane and internalize it for degredation by lysosymes[8, 9]. However, it has been demonstrated that the binding sites for M6P and IGF-II are distinct[10]. This putative function of regulating levels of IGF-II has important implications not only for normal development processes but also for mitogenesis.

The receptor has also been demonstrated to play a role in the activation of transforming growth factor beta (TGFβ) from its latent precursor found in the extracellular matrix[11]. Latent TGFβ (LTGFβ) binding to the CI-M6P/IGF-II receptor has been previously demonstated[12]. Related to this function the receptor has also been demonstrated to interact with non-M6P containing ligands: plasminogen, urokinase-type plasminogen activator receptor (uPAR), and proliferin[13-15]. The complex of proliferin and the CI-M6PR cleaves the LTGFβ. The direct binding of uPAR enhances this reaction. The effects of TGFβ on cells is complex ranging from growth inhibition of normal cells to paradoxically both suppression and promotion of tumour cells[16]. In part TGFβ influences the cell cycle and induces growth arrest by altering the expression of cyclin D kinase inhibitors p15 and p21[17, 18]. However, in tumourigenesis TGFβ has several roles including: promotion of tumour growth, invasiveness and angiogenesis, development of resistance to apoptosis, and suppression of immune effector response[19]. The fact that the CI-M6PR plays an important role in regulation and activation of TGFβ which in turn has so many influences on cell functions important in development, migration, angiogenesis, and immune modulation suggests it may also have a significant function in early and late carcinogenesis.

Retinoic acid is an essential growth factor derived from vitamin A. It is recognized to have an important role in development of many organ systems especially central nervous system embryonic growth and differentiation[20, 21]. It also has influence on the adult brain[22]. The influence of retinoic acid is mitigated by induction of various growth factor receptors such as: transforming growth factor receptor[23], epidermal growth factor receptor[24], and platelet derived growth factor[25]. Interestingly retinoic acid has also been identified as a ligand of the CI-M6PR[26].

It is not a surprise then with so many important molecules interacting with the CI-M6PR that it has been found to be developmentally regulated. Receptor levels in rats peak near term and diminish rapidly postnatally[27]. In mice that are homozygous for a mutation disrupting the CI-M6PR, the offspring are small but of normal proportion[28, 29]. Furthermore, 100% fetal lethality was found in IGF-II receptor null mice [30]. It was proposed that the cause was over-expression of IGF-II since creation of an IGF-II null allele allowed survival of the receptor-null offspring. The role of the receptor in embryologic development may be important in specific organ systems. The predominant region of CI-M6PR expression in chicken embryos is the developing myocardium and is paramount to normal mature architecture [31].

The CI-M6PR has also been studied in regard to nervous system development. Using radiolabelled IGF-II, binding to the CI-M6PR has been shown to have a predominantly cortical distribution by 25 weeks of gestation in the human fetus[32]. This wide cortical distribution is continued in adult human brains with the highest IGF-II specific binding occurring in the cingulum, hippocampus, and hypothalamus[33]. In rats after 3 weeks of age the IGF-II receptor was detected in neurons throughout the forebrain with greatest levels in the hippocampus[34]. The significant regulatory role of IGF-II in normal brain development is further suggested by an observation of 10 times the normal levels of IGF-II in a 3 month old with macrocephaly [35]. In addition, cultures derived from fetal rat brains that were treated with IGFs demonstrated significant increases in DNA synthesis[36]. It is important to note that in studies of normal human brain and cerebral spinal fluid, IGF-II and its precursors were detected but IGF-I was not[37]. Using a polyclonal anti-serum against the CI-M6PR, Valentino et. al. demonstrated high levels of receptor expression in the developing brain especially the cortex and hypothalamus. Receptor expression declined by post-natal day 7 and was maintained at those levels into adulthood. This study made particular note that CI-M6PR immunoreactivity was detected to extend from the ventricular surface to the marginal zone at the pial surface[38]. The authors also point out that CI-M6PR levels were very high and localized in developing skin. These observations as well as the demonstration that IGF-II binds to both IGF-I receptor and CI-M6PR in cortical membranes from human and rat brains[39] consolidate the important role of IGF-II and its receptors in brain growth and development.

As the above discussion indicates the CI-M6PR is intrinsically involved in important normal development processes. However, the observation that the CI-M6PR controls activation of the growth inhibitor TGFβ and the internalization and degradation of IGF-II, also suggests the potential for influence as a mitogen. Several observations in various cancer types support this idea.

The prevalence of altered CI-M6PR function in cancer has best been described in hepatocellular tumours. Up to 70% of human hepatocellular tumours demonstrate loss of heterozygosity (LOH) of the CI-M6PR gene[40]. This is an early event in carcinogenesis and is consistent across various populations[41, 42]. Interestingly, one group has demonstrated an insertion sequence in the gene that results in truncation of the CI-M6PR protein at the region responsible for the transmembrane domain and lysosomal trafficking signal[43]. Similarly microsatellite instability leading to frameshifts and premature stop codons in the CI-M6PR sequence has been shown to be prevalent in gastrointestinal tumours[44]. Receptor LOH has also been identified early in breast carcinomas and squamous cell carcinomas of the lung[45, 46]. Furthermore, the functional capacity of the receptor has been correlated with tumour progression in hepatocellular cancer, breast tumours, choriocarcinoma, and rhabdomyosarcoma cells[47-50].

The ability of cancer cells to avoid immune surveillance and cytotoxic T-lymphocyte (CTL) mediated death is one of the five hallmark characteristics of cancers[51]. CTLs rely on four main strategies to kill their targets. These include, release of cytotoxic cytokines such as tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), apoptotic signal transduction mediated by binding of Fas ligand with Fas receptor on target cells, Granzyme mediated apoptosis[52] and TNF related apoptosis-inducing ligand (TRAIL)[53]. Of these strategies, immune effector cells including CD8+

CTLs, natural killer cells (NK), and lymphokine activated killer T-cells (LAK) have been shown to depend primarily on the perforin/granzyme B system to kill their targets[54-56]. Importantly, it has recently been demonstrated that the CI-M6PR at least in part mediates the internalization of granzyme B into target cells[57]. Once internalized, granzyme B can act via two different paths. It can cleave and activate caspase 3 leading to target cell apoptosis[58]. It can also act via the mitochondria by cleavage of BID that ultimately results in mitochondrial disruption and cell death by necrosis[59, 60]. Clearly then, cancer cells that bear mutations of the CI-M6PR may have the advantage over the immune system by avoiding the target effects of granzyme B.

The purpose of this study is to explore the role the CI-M6PR may play in tumour insensitivity to granzyme B mediated apoptosis in two important tumour types affecting the central nervous system: high grade astrocytomas and metastatic melanomas. These tumours, both of neuroectodermal origin, are aggressive and uniformly lethal tumours despite all currently available treatment modalities. It is well recognized that both of these tumour types have evolved complex mechanisms to evade immune system recognition and killing responses. It has been shown that cytotoxic T-lymphocytes can infiltrate gliomas but are unable to function as effector cells[61-63]. Similarly ineffective tumour infiltrating lymphocytes and circulating antigen specific T-lymphocytes have been identified in melanoma patients[64, 65]. In both tumour types the cytokine milieu in the microenvironment and modulation of immune effectors have been shown to be contributory to immune system evasion[66-68].

Previous investigations have suggested an association of the CI-M6PR with gliomas and melanomas. For gliomas increased expression of IGFs and IGF receptors has been demonstrated[69-72]. A single study of microsatellite instability in gliomas revealed a frame shift mutation in the CI-M6PR in 1 out of 22 patients with high grade tumours[73]. In melanomas LOH on chromosome 6 was found to be a frequent event observed in tumour samples from 20 patients[74]. These observations suggest an important role that alteration in CI-M6PR expression may play as either a mitogen or as an immune modulator. As yet no study of gliomas and melanomas has explored the expression of the CI-M6PR and the functional impact of this receptor with respect to mediating granzyme B induced apoptosis.

In this investigation we report that cells from early explant cultures derived from gliomas and melanomas as well as established tumour cell lines demonstrate highly variable expression of the CI-M6PR. We identify a glioma tumour in which significantly elevated receptor expression is also associated with functional abnormalities. In particular these cells demonstrated both an inability to internalize granzyme B and an insensitivity to granzyme B mediated apoptosis. Our observations indicate that abnormalities involving the CI-M6PR play a pivotal role in some glioma and melanoma cells resistance to CTL mediated death.

Methods:

Evaluation of cell surface expression of the CD- and CI- M6PR.

Table 4.1 lists the tumour cell lines and early explant tumour cells evaluated for expression of the M6PR. Cells were grown on low surface binding tissue culture plastic (Sarstetd 83.1813.502). Cells were harvested by incubation with 2mM EDTA in PBS at 37°C. The cells were rinsed in cold phosphate buffered saline (PBS) with 0.1% bovine serum albumin (BSA) to remove all culture media containing serum. $1x10^6$ cells were suspended PBS with 0.1% BSA in polypropylene microcentrifuge tubes and maintained at 4°C. The cells were then incubated in primary antibodies for 20 minutes. We used the anti- CI-M6PR rabbit anti-bovine antibody (generous gift from Dr. WJ Brown), and anti- (CD)-M6PR rabbit anti-human antibody (generous gift from Dr. WS Sly). Following the primary incubation, the cells were washed twice with 0.1% BSA in PBS. Centrifugation for 5 minutes at 1000 rpm was utilized for cell recovery. The cells were then incubated for 30 minutes at 4°C with goat anti-rabbit Cy2 secondary antibody (Jackson 111-225-1440). After incubation with secondary antibody cells were washed twice with 0.1% BSA in PBS and finally re-suspended in 500µl PBS with 1% formaldehyde. Tumour cells were evaluated for the expression of the M6PR in a variety of early explant gliomas and melanomas relative to normal and transfected control cell lines using a Becton Dickison flow cytometer and Cell Quest software. We also evaluated Granzyme B fluorescently labeled with Oregon green (GrB-OG) in some cell lines (generous gift from Dr. C Bleackley).

Controls	Tumour cell lines	Melanoma explants	Glioma explants
L cells	SK Mel 24	Ed 235 Mel	Ed 189 BT
MS	SK Mel 28	Ed 178b Mel	Ed 273b BT
MS 9-II	LN 18		
SR 2-I	LN 71		
Jurkat	LN 229		
N 30	U 251		
N 39	U 343		
N 42	U 54 .mg		
	T 98 G		

Table 4-1 Cell lines evaluated by flow cytometry for surface expression of the CI-M6PR and CD-M6PR. See figures 1&2 for results.

Evaluation of CI-M6PR localization with confocal microscopy.

Tumour cells were harvested as detailed above and then fixed with 2% paraformaldehyde. The cells were then incubated in primary antibodies for 20 minutes. We used the anti- CI-M6PR rabbit anti-bovine antibody. Following the primary incubation, the cells were washed twice with 0.1% BSA in PBS. The cells were then incubated for 30 minutes at 4°C with goat anti-rabbit Cy2 secondary antibody (Jackson 111-225-1440). The cells were transferred to glass slides allowed to dry briefly then #1 coverslips were mounted with glycerol.

To evaluate dendritic cells the cells were grown on glass (Corning #1) coverslips (NUNC, Lab-Tek® precoated 8 chamber slides) additionally coated with 1% D-Ploy-L-lysine (Sigma). Fixation and immunohistochemistry for CI-M6PR was identical to above. A second primary antibody mouse anti-CD83 was used to confirm mature DC phenotype. This was visualized using the secondary goat anti-mouse Cy3. Confocal imaging was performed with a Ziess Axiovert 100M LSM 510 laser scanning microscope.

Evaluation of CI-M6PR mediated uptake of GrB-OG.

Tumour cell lines and control cells were grown in their optimal media in standard conditions. Cells were harvested from tissue culture flasks using 2 mM EDTA in PBS and counted to obtain $2x10^6$ cells per ml. Cells were washed twice with PBS containing 0.1% BSA to remove serum that had been present in the growth and support media. The cells were plated at a density of $2x10^5$ cells per experimental and control well in a polypropylene V-bottom MTP. A single plate was used for each experimental group (ie. each time and temp point). Cells were left to recover from harvest for one hour in an incubator, humidified at 37°C and 5% CO_2.

After the recovery period the V-bottom polypropylene plates were spun down for 5 minutes at 300xg and the supernatant gently removed. Cells were then suspended with 23uL of Dulbecco's modified Eagle's media (DMEM) and 2uL(25ng) of granzyme B tagged with the fluorochrome Oregon green. The GrB-OG was a generous gift from Dr. C. Bleackley. This labeling step was performed on ice. Note that the DMEM was supplemented with 0.1% BSA, 0.2 mM HEPES buffer, L glycine, and pH established between 6.5 and 7.2.

The control plates for all cell lines were kept at 4°C. The GrB uptake plates were transferred to an incubator and kept at 37°C for 15 or 60 minutes in the dark. At the completion of incubation times all plates were maintained on ice. 150uL of supplemented cold DMEM media was added to each well. Plates were spun down for 5 minutes at 300xg and maintained at 4°C. The supernatant was gently removed. Next 180uL of PBS with 0.1% BSA was added and plates spun again. The supernatant was removed and cells fixed by adding 400ul of 4% paraformaldehyde. Cells were then transferred to FACS tubes and kept shielded from light with foil. Samples were evaluated using a Becton Dickison flow cytometer (channel FL1 at around 520nm - excitation occurs at 494nm and emission at 519nm) and Cell Quest software.

Evaluation of GrB mediated apoptosis using TUNEL assay.

Tumour cell lines and control cells were grown in their optimal media in standard conditions until required for further studies. Cells were harvested from tissue culture flasks using 2 mM EDTA in PBS and counted to obtain $2\text{x}10^6$ cells per ml. Cells were washed twice with PBS containing 0.1% BSA to remove serum that had been present in the growth and support media. The cells were plated at a density of $2\text{x}10^5$ cells per experimental and control well in a polypropylene V-bottom MTP. Cells were left to recover from harvest for one hour in an incubator, humidified at 37°C and 5% CO_2.

After the recovery period the V-bottom polypropylene plate was spun down for 5 minutes at 300xg and the supernatant gently removed. Cells were then suspended in 75 μl of DMEM/F12 media supplemented with 0.1% BSA. GrB was then added and cells incubated humidified at 37°C and 5% CO_2 for 1 hour. 30 pfu's of adenovirus were then added to the appropriate wells and incubation continued for 3 hours.

The cells were then prepared for evaluation of DNA fragmentation by TUNEL assay. The technique used was as per the company instructions (Roche Diagnostics). The cells were evaluated for TUNEL positivity by flow cytometry. The percent specific TUNEL cells was calculated as: [(% positive labeling cells with GrB and AD - % positive labeling cells without GrB and AD)/ 100 - % positive labeling cells without GrB and AD)] x 100.[75] The same method of evaluation was used for CD8+ T lymphocytes and dendritic cells.

Evaluation of GrB and CTL mediated apoptosis using ^{3}H-Thymidine release assay.

Tumour cell lines and control cells were grown in their optimal media in standard conditions until required. Cells were grown to confluence in T75 tissue culture flasks. Media was changed and the cells bathed in 30 ml of media with 30 μCi of ^{3}H-thymidine. The cultures were then incubated overnight or approximately 1 cell cycle at 37°C, 5% CO_2 in a radiation certified incubator. Cells were washed with warm PBS and then harvested using trypsin/ EDTA for 2 minutes at 37°C. The cells were recovered with 10 ml of media and transferred to a 50 ml centrifuge tube. The cells were washed twice with media resuspended in media and allowed to recover for 1 hour in the incubator.

The ^{3}H-thymidine loaded cells were then challenged with either GrB (600ng/ml) and AD (30 pfu per cell), or admixture with cytotoxic t-cells at a 5:1 effector to target ratio. In both cases the incubation time was 3 hours. Following incubation SDS-NaOH lysis buffer was added to the cells and the sample was vortexed in order for the cells to lyse and release DNA fragments. The samples were then centrifuged in order to pellet the cellular debris and intact DNA. The supernatant, containing the labeled DNA fragments, was collected and the amount of radioactivity (proportional to the amount of apoptotic cell death) determined. Radiation counts were performed on a Beckman LS β-radiation counter.

Evaluation of caspases and anti-apoptotic proteins.

For western blot analysis tumour cell cultures were grown in 75 ml tissue culture flasks and harvested when subconfluent. The cells were then lysed in NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris (pH 8.0)) supplemented with fresh 1mM phenylmethoxylsulfonyl fluoride and protease inhibitor cocktail (SIGMA). The lysates were centrifuged at 14,000 x g to remove cellular debris. Each lysate was then evaluated for protein concentration using the Bio-Rad Dc Protein Assay Kit (Bio-Rad Laboratories, California). Equal amounts of protein, 20 μg, from each tumour were separated through Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane (Bio-Rad), and blocked with 5% nonfat dry milk in Tris-Tween Buffered Saline (TTBS, 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 0.5% Tween 20) at room temperature for 1 hour. The membranes were then incubated overnight at 4°C with the particular primary antibody diluted in buffering solution (TTBS and 0.5% bovine serum albumin). Primary rabbit anti-human antibodies to: caspase 3 (diluted 1:5000), cellular FLICE-like inhibitory protein (cFLIP) (1:1000), FADD (1:1000), Survivin (1:1000), XIAP (1:1000), extracellular signal – regulated kinases 1 and 2 - ERK 1&2 (1:1000) were purchased from SressGen Biotechnology Corp.(Victoria, British Columbia). Phosphoprotein enriched in Astrocytes-M_r 15000 (PEA15) (1:1000) was kind gift of Dr. C. Hao (Department of Laboratory Medicine, Neuropathology, University of Alberta). Mouse anti-human caspase-8 monoclonal antibody C15 was a kind gift from Dr. Marcus Peters (German Cancer Research Centre, Heidelberg, Germany).

The membrane was washed and then incubated with peroxidase-conjugated goat anti-rabbit or rabbit anti-mouse antibody (1:5000, from Jackson Immunoresearch Laboratories Inc, Pennsylvania) for 1 hour. The blots were then washed and developed by chemilumminescence according to the manufacturer's protocol (NEN Life Sciences Products Inc, Massachusetts)

Evaluation of the CI-M6PR sequence.

RNA isolation was performed using Trizol® reagent (Invitrogen). RNA was purified from approximately 5.0 x 10^6 cells of the tumour lines Ed 273b BT and Ed 189 Bt using the method supplied by the manufacturer. The concentration of RNA was assessed fluorometrically using ethidium bromide.

RT-PCR was performed using the Superscript™ III One-Step RT-PCR system (Invitrogen). 0.5 µg of total RNA was first reverse transcribed into first strand cDNA then subsequently amplified with gene specific primers according to manufacture's instructions. Initial annealing temperature for cDNA synthesis was 50°C. PCR reaction conditions were as follows: 35 cycles of 94°C - 30 seconds, 52°C – 30 seconds, 72°C – 45 seconds, and a final extension step at 72°C for 10 minutes. Equal amounts of PCR product were electrophoresed on a 0.9% agarose gel and visualized by ethidium bromide staining.

Northern blot analysis. Total RNA was separated on a denaturing formaldehyde agarose gel and transferred onto a Hybond™- N nylon membrane (Amersham) by capillary transfer. The blot was UV cross-linked and pre-hybridized using ULTRAhyb™ (Ambion) at 45°C. The blot was subsequently probed with α32P dCTP using the Random Primer DNA Labeling System (Invitrogen). After and overnight hybridization at 42°C, the blot was washed and exposed with a phosphoimager plate.

Results:

The data revealed highly variable cell surface expression of the CI-M6PR in glioma and melanoma cultured tumour cells – [**Figure 4-2**]. Mann-Whitney U- test pairwise comparison indicated significant CI-M6PR expression differences compared to MS (murine control cell with low CD and CI-M6PR expression) for: SK Mel 28 $p=0.034$ (ATCC tumour line), LN 18 $p=0.02$, and Ed 189 Bt $p=0.013$ (early glioma explant). By Kruskal Wallace one-way ANOVA there was no significant differences in surface expression of the CD-M6PR, $p \leq 0.05$ in the same tumours [**Figure 4-3**].

A comparison of early explant glioma cells using confocal microscopy demonstrated distinct distributions of the CI-M6PR [**Figure 4-4**]. In Ed 273b Bt cells the receptor showed typical punctuate, peri-nuclear localization of the receptor. In contrast Ed 189 Bt revealed a much more diffuse cytosolic distribution.

On the basis of these interesting findings of variable receptor expression and differential localization among various tumours we then evaluated the functional quality of the CI-M6PR with respect to internalization of GrB. We directly compared

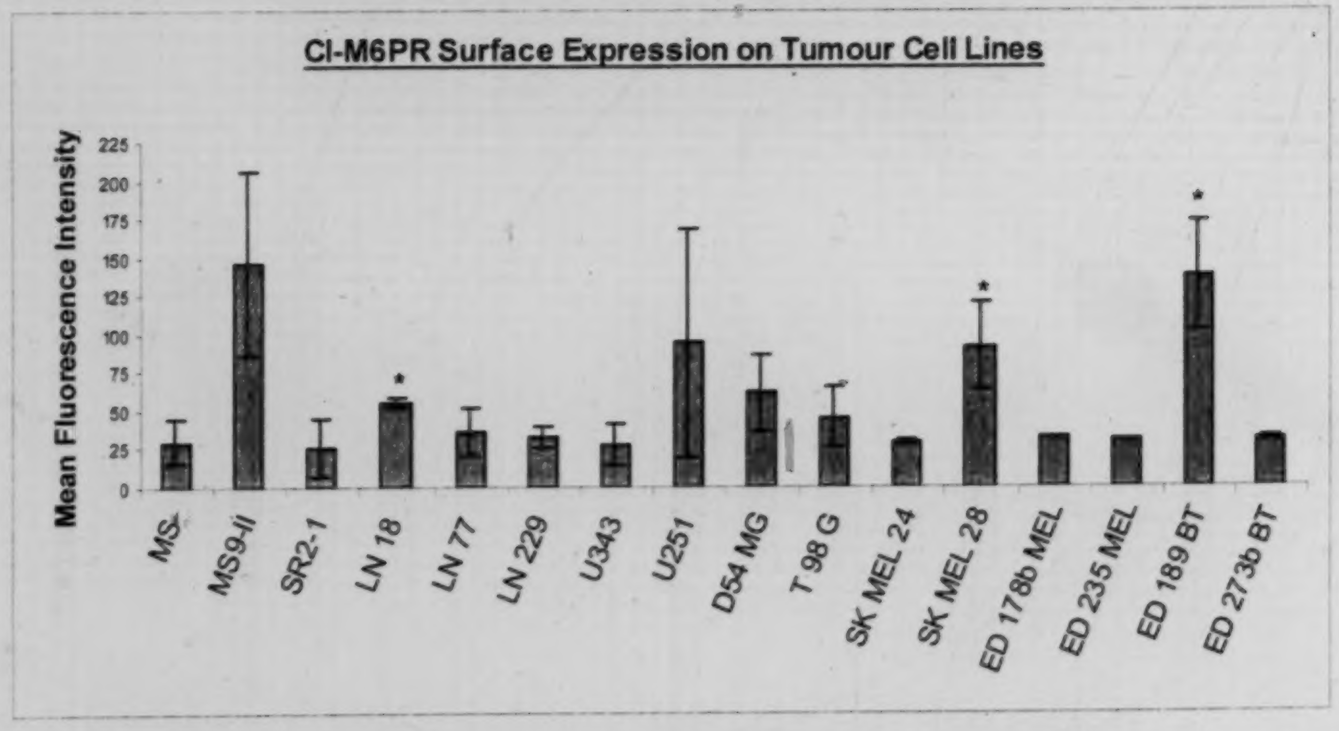
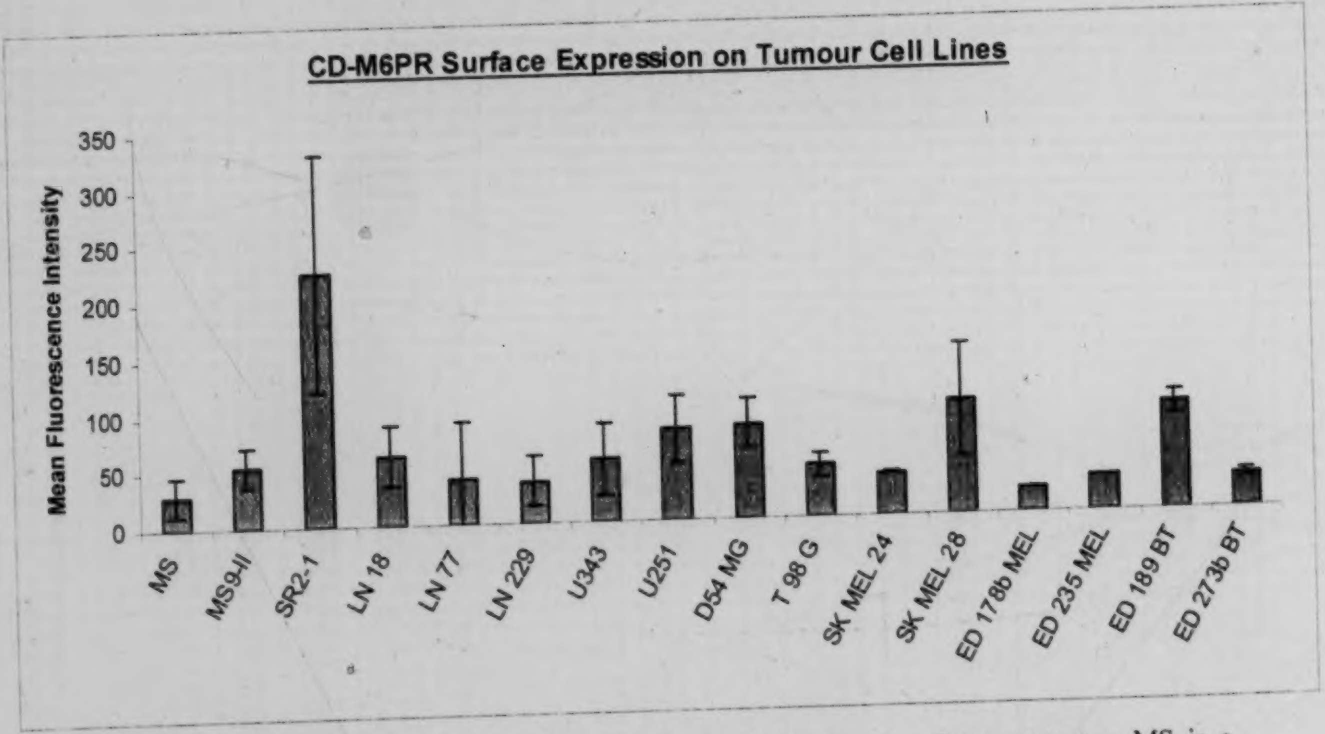


Figure 4-2 Relative mean fluorescence intensity as determined by flow cytometry. MS is a murine control line with low CD and CI-M6PR expression. MS 9-II is a transfected MS line to over express the CI-M6PR. Jurkat cells are a human lymphocyte line. * indicates significant values $p < 0.05$.



CD-M6PR Surface Expression on Tumour Cell Lines

Figure 4-3 Relative mean fluorescence intensity as determined by flow cytometry. MS is a murine control line with low CD and CI-M6PR expression. MS 9-II is a transfected MS line to over express the CI-M6PR. SR2-1 is a transfected MS line to over express the CD-M6PR. Jurkat cells are a human lymphocyte line. * indicates significant values $p< 0.05$.

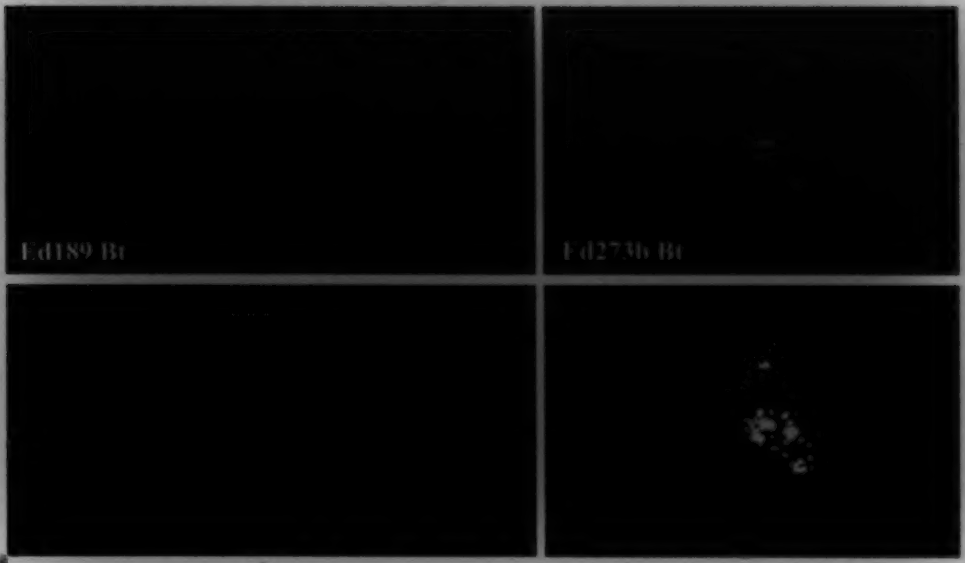


Figure 4-4 Confocal Images of CI-M6PR Localization

Localization of the CI-M6PR for high grade glioma tumour explant cells, Ed 189 Bt & Ed 273b Bt . The primary antibody - rabbit anti-bovine CI-M6PR (generous gift from Dr. WJ Brown) was visualized using the secondary goat anti-rabbit CY5. Images acquired using Zeiss LSM 510 confocal microscope stimulating with 488nm laser.

GrB-OG internalization in MS (negative control), Jurkat cells (positive control), early explant gliomas: Ed 273b Bt (low surface receptor expression) and Ed 189 Bt (high surface receptor expression), as well as melanoma lines: SK Mel 24 (low surface receptor expression) and SK Mel 28 (high surface receptor expression). We found that for MS and Ed 189 Bt there was no internalization of the GrB-OG as determined by flow automated cytometric stream (FACS) [**Figure 4-5**]. The other cell lines all demonstrated progressive accumulation at 15 and 60 minutes of incubation.

Due to the observation in Ed 189 Bt that elevated surface expression was associated with poor internalization of GrB we investigated the induction of apoptosis following incubation with GrB and AD in all cell lines [**Figure 4-6**]. DNA fragmentation as determined by TUNEL labeling of cells revealed significant apoptosis induced in Jurkat and Ed 273b Bt cells. There was minimal response in the melanoma cell lines and no specific TUNEL positivity at any dose of GrB for Ed 189 Bt.

We used western blot analysis to evaluate if key elements of the apoptotic pathways were present in the cells being compared [**Figure 4-7**]. All tumour cells expressed Caspase 8, Caspase 3, and Fas associated death domain (FADD). ERK 1&2 expression was used as a control. Interestingly we found that there was low level expression of recognized anti-apoptotic proteins XIAP, cFLIP, Survivin, and PEA 15 in each of the tumour lines [**Figure 4-8**].

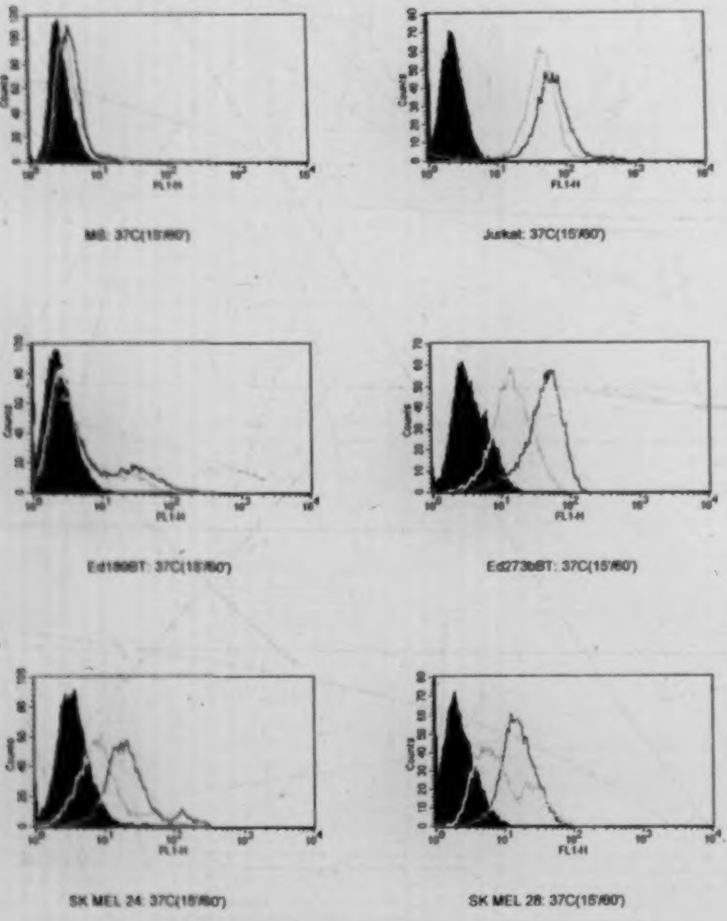


Figure 4-5 Evaluation of GrB-OG uptake.

Cells were incubated with 25ng of GrB-OG for 15 or 60 minutes at 37ºC open peaks. Solid peak is the control – no GrB-OG. MS is murine negative control. Jurkat are human lymphocyte positive control cell line.

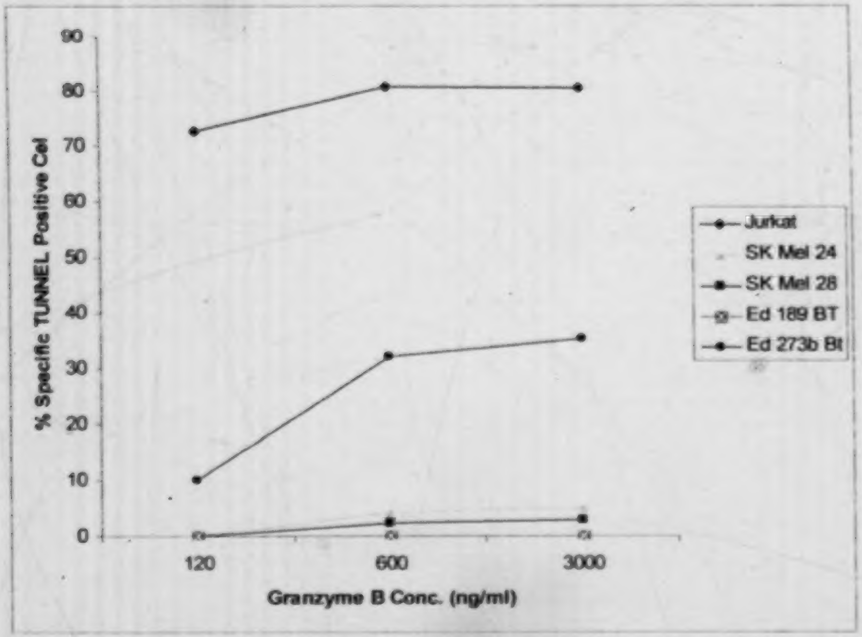


Figure 4-6 TUNEL Assay of GrB Mediated Apoptosis of Tumour Cells

Granzyme B mediated DNA fragmentation. The percent specific positive TUNEL labeling of cells after 3 hour incubation with GrB and AD was determined by flow cytometry.

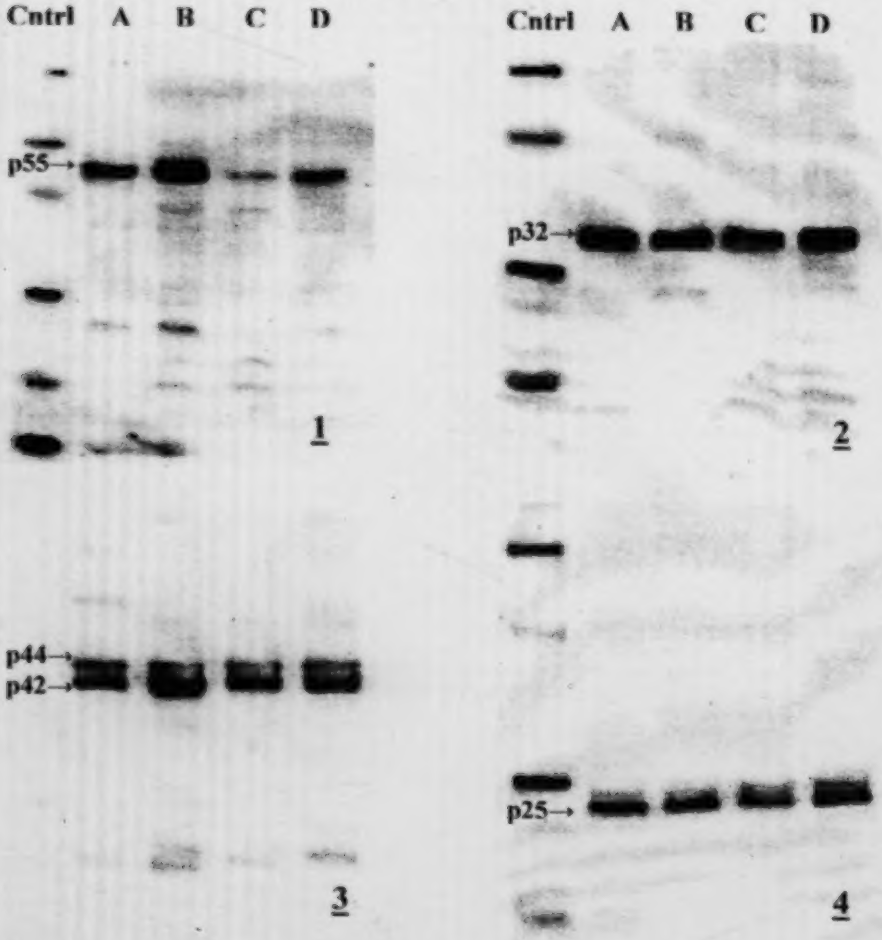

Cntrl
A
B
C
D
Cntrl
A
B
C
D
p55→
p32→
1
2
p44→
p42→
p25→
3
4

Figure 4-7 Western blot detection of tumour cell line apoptosis related proteins. A = SK Mel 24, B = SK Mel 28, C = Ed 189 Bt, D = Ed 273b Bt. **1.** caspase 8. **2.** Caspase 3. **3.** ERK 1&2, **4.** FADD.

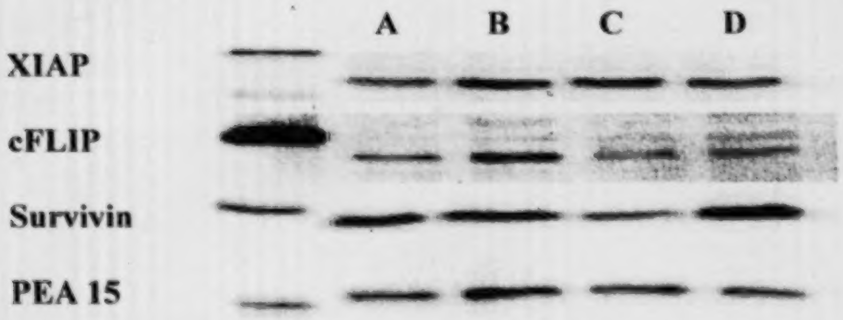

A
B
C
D
XIAP
cFLIP
Survivin
PEA 15

Figure 4-8 Western blot evaluation of tumour cell line expression of anti-apoptotic proteins. A = SK Mel 24, B = SK Mel 28, C = Ed 189 Bt, D = Ed 273b Bt.

Reverse transcription PCR was employed using primers targeted to the transmembrane and cytoplasmic tail of the CI-M6PR to evaluate the possible presence of a sequence mutation in this region. We compared the two early explant gliomas Ed 273b Bt and Ed 189 Bt and found no sequence abnormality.

We were also interested in determining if there were differences in CI-M6PR expression and function in important anti-tumour immune cells. In particular we investigated normal human CD8+ CTLs and DCs. We found that there was negligible surface expression of the CI-M6PR on CD8+ CTLs. Furthermore, there was no difference in the surface expression between either CD69+ (activated) or CD69- (naïve) CD8+ lymphocytes [**Figure 4-9**]. Low levels of surface expression for the receptor were noted in both immature and mature DCs [**Figure 4-10**]. However, there was no significant difference with respect to the mean fluorescence intensity. Confocal imaging of immature DCs revealed a predominantly peri-nuclear distribution of the CI-M6PR [**Figure 4-11**], whereas, in the mature DCs there was a more dispersed distribution throughout the cytosol [**Figure 4-12**]. A three dimensional reconstruction study confirmed that there was no significant change in surface receptor concentration (data not shown). Finally, we evaluated whether or not there was a difference in vulnerability to GrB mediated apoptosis between the immature and mature forms of the dendritic cells. Following incubation with 300 ng/ml or 600 ng/ml of GrB in the presence of AD, no apoptosis was detected [**Figure 4-13**]. Even at 3000 ng/ ml of GrB neither CD8+ lymphocytes nor DCs underwent apoptosis (data not shown).

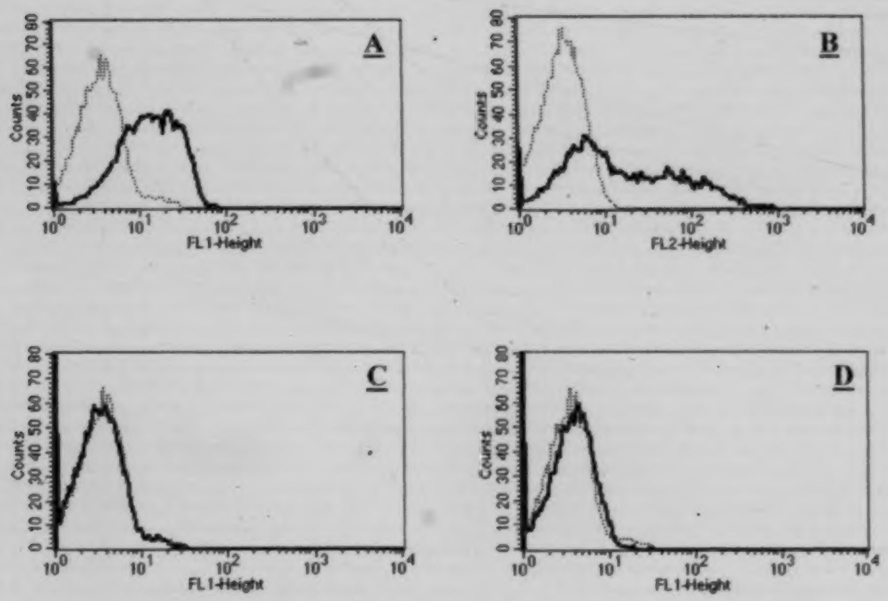


Figure 4-9 CTL Expression of the CI-M6PR

Evaluation by flow cytometry of surface expression of the CI-M6PR by normal human cytotoxic T-lymphocytes. **A** confirmation of CD8 expression. **B** CD8+ cells express CD69 after 24 hour incubation with PHA to cause activation. **C** Surface expression of CI-M6PR by naïve CD8+ cells. **D** Surface expression of CI-M6PR by activated CD8+ lymphocytes. Bold peaks represent experimental sample detection.

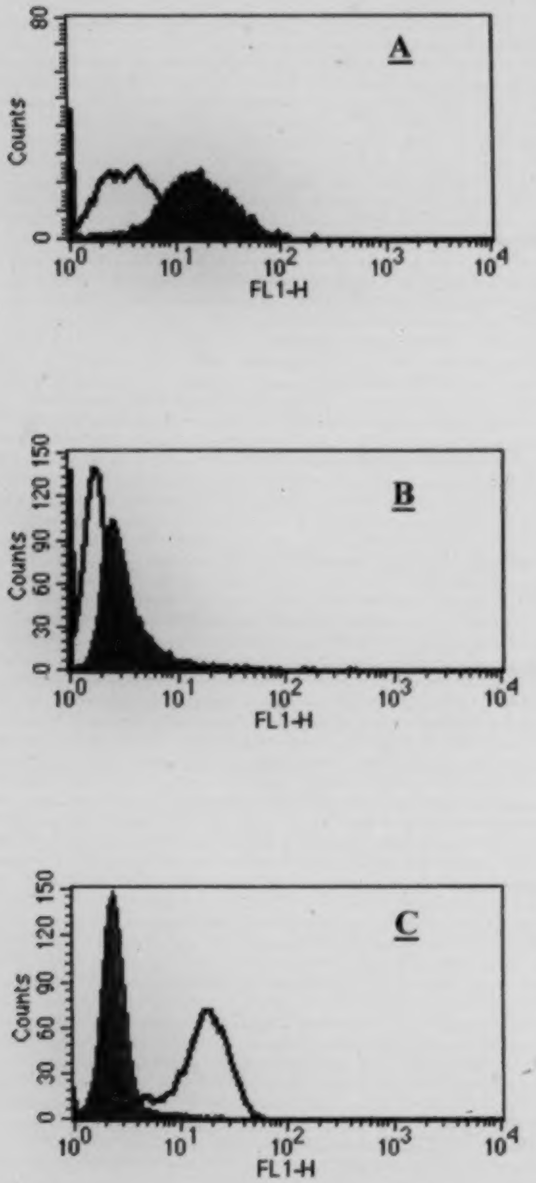


Figure 4-10 **DC Expression of the CI-M6PR**

Evaluation by flow cytometry of surface expression of the CI-M6PR by **A** immature, and **B** mature normal human dendritic cells. Expression of CD83 was used to confirm mature dendritic cell profile **C**. Shaded peaks represent controls for fluorochrome detection.

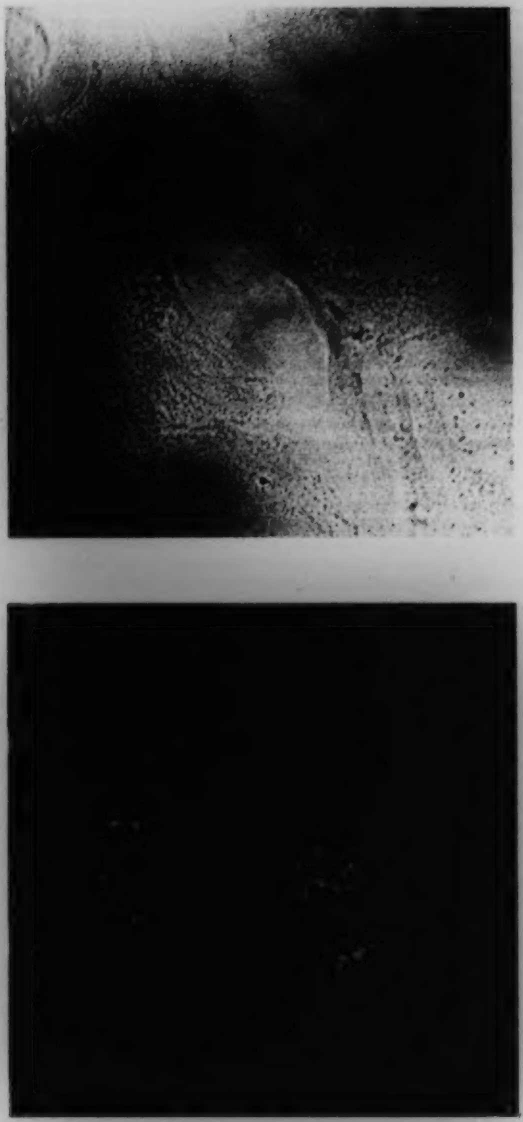


Figure 4-11 Confocal Images of CI-M6PR Localization in Immature DCs

Localization of the CI-M6PR in immature normal human dendritic cells. The primary antibody - rabbit anti-bovine CI-M6PR (generous gift from Dr. WJ Brown) was visualized using the secondary goat anti-rabbit CY5. Images acquired using Zeiss LSM 510 confocal microscope stimulating with 488nm laser.

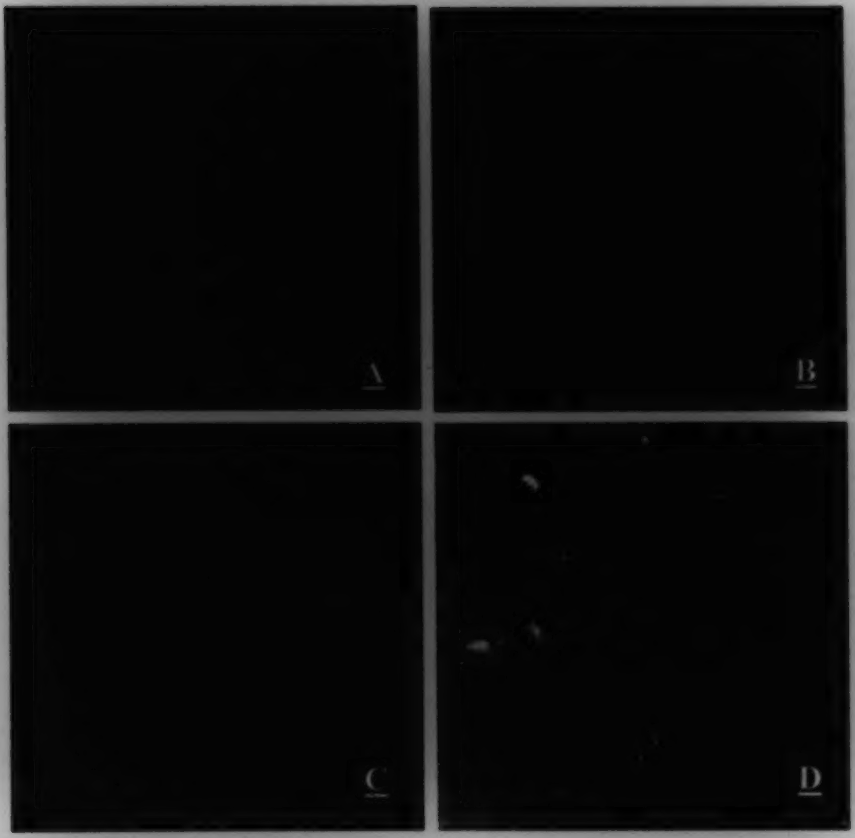


Figure 4-12 Confocal Images of CI-M6PR Localization in Mature DCs

Localization of the CI-M6PR in mature normal human dendritic cells. **A:** The primary antibody - rabbit anti-bovine CI-M6PR (generous gift from Dr. WJ Brown) was visualized using the secondary goat anti-rabbit CY5. **B:** The primary antibody mouse anti – CD83 was visualized using the secondary goat anti-mouse CY3. **C:** Is a composite overlay of **A** and **B.** **D:** is a phase contrast image. Images acquired using Zeiss LSM 510 confocal microscope.

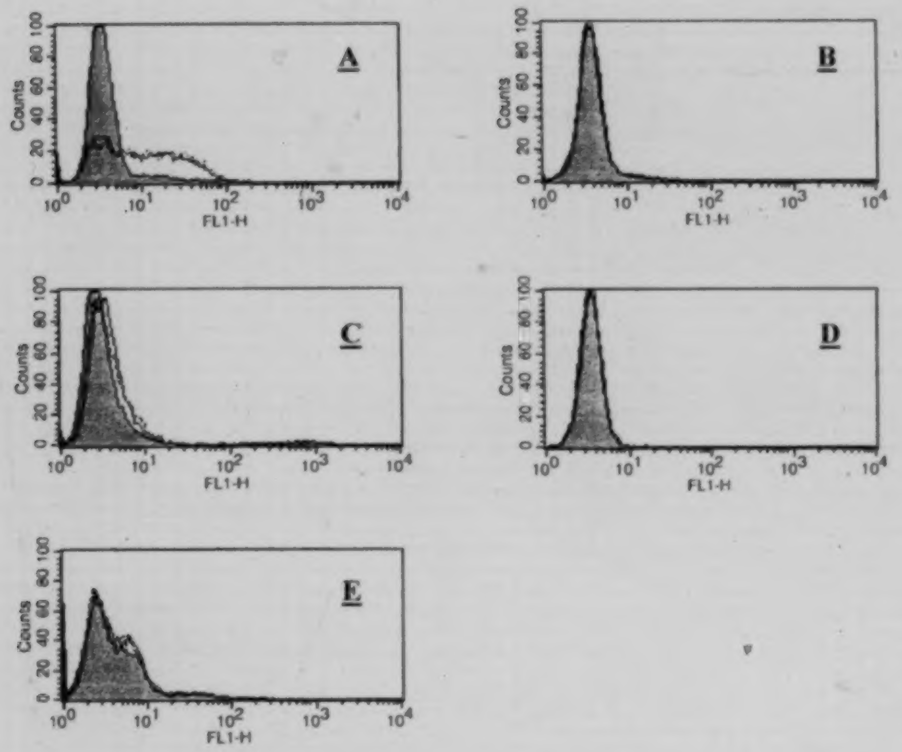


Figure 4-13 TUNEL Assay of GrB Mediated Apoptosis of CTLs and DCs

Evaluation by flow cytometry of TUNEL expression following 3 hour incubation with 300ng/ml or 600ng/ml of GrB + AD. **A** Jurkat cells are positive controls. **B** CD8+, CD69- normal human lymphocytes. **C** CD8+, CD69+ normal human lymphocytes. **D** Normal immature human DCs. **E** Normal mature human DCs. Shaded peaks represent controls for fluorochrome detection.

Discussion:

In this study we were interested in determining if there are firstly differences in expression of, and secondly differences in the function of the M6PR in established cell lines and early explant cultures of glioma and melanoma tumours. We found that 2/9 gliomas (1/7 tumour cell lines, 1/2 early explant cultures), and 1/4 melanomas (1/2 cell lines) demonstrated significantly elevated surface expression of the CI-M6PR.

Previously, a study comparing I^{125}-IGF-II binding to membranes isolated from both fresh cadaveric brains and fresh glioblastoma tumour samples revealed significant increase in IGF-II binding to the tumour membranes compared to normal brain tissue[69]. Gammeltoft and colleagues found that membrane binding of I^{125}-IGF-II to membranes isolated from 6 glioma cell lines was similar to that of cultured fetal rat astrocytes but elevated compared to membranes from adult rat cortex[71]. Finally, using *in situ* hybridization with cDNA probes for the CI-M6PR, Antoniades demonstrated mRNA expression for the receptor in astrocytoma samples but not in human cortex[72]. No similar studies have been performed looking at CI-M6PR expression in melanomas. Significantly, none of the above investigators have explored the functional implications of these findings with respect to either tumourigenesis or immune system avoidance.

In our study we present a comparison of selected melanomas and gliomas with respect to their functional ability to internalize GrB and vulnerability to GrB mediated apoptosis. What our study has demonstrated is that for at least one tumour early explant line, that with the highest surface receptor expression, internalization of GrB was negligible and further, that incubation with GrB + AD or activated CTLs did not induce apoptosis. The first evidence for CI-M6PR function as a cell membrane receptor and transporter into the cytosol was provided by Oka et.al. Using techniques of membrane fractionation they demonstrated I^{125}-IGF-II receptor binding and internalization by adiposites[76]. In addition, these investigators demonstrated that membrane bound receptor concentration and trafficking could be enhanced by various agents especially insulin and retinoic acid (RA)[9, 77]. Subsequent to that, Motyka and colleagues determined that GrB internalization was also mediated by the CI-M6PR, and that GrB binding to the receptor and induction of apoptosis could be competitively inhibited by M6P[57]. Furthermore, transfected cells that overexpressed normal CI-M6PR showed enhanced vulnerability to killing by GrB + AD in comparison to normal control cells that were receptor negative. In contrast, our studies show that tumour cells expressing high levels of CI-M6PR, such as Ed 189 Bt, did not demonstrate enhanced uptake and killing by GrB. This important finding suggests that tumour cell resistance to cytotoxic T-lymphocyte killing may be mediated through intrinsic abnormalities in the CI-M6P receptor.

Under normal conditions, the CI-M6PR continuously cycles to the cell surface membrane with the vast majority of receptor proteins, > 90%, located in the perinuclear region and associated with intracellular transport of lysosymes[78]. The important role of the CI-M6PR binding ligands at the cell suface and internalizing them has been well demonstrated. Using radiolabelled IGF-II, internalization and intracellular degradation was shown[8]. In addition to internalization of GrB, surface binding and internalization has also been shown for RA[26, 77] and exogenous β-glucuronidase[79, 80].

Lobel et. al. identified residues in the cytoplasmic tail region that are responsible for CI-M6PR endocytosis capacity[81]. Using murine L-cells deficient in endogenous CI-M6PR, the authors investigated the function of transfected bovine CI-M6PR cDNA with various cytoplasmic domain deletions. They found that the inner portion of the cytoplasmic tail is necessary for receptor endocytosis. Failure of endocytosis was evidenced by accumulation of the receptor on the cell suface. Further experiments using the same strategy of serial truncated mutations of the receptor into L-cells specifically identified residues 26-29 of the cytoplasmic tail as being the essential moiety for endocytosis[82, 83]. In an hepatocellular carcinoma that was found to have a large insertional mutation resulting in a truncated CI-M6PR affecting the transmembrane domain, investigators demonstrated accumulation of the receptor specifically to the cell membranes and immediate extracellular spaces[43].

It is recognized that in normal humans fragments of the CI-M6PR may be detected in serum and urine and that shedding from the membrane may represent the normal physiologic state for turnover of the receptor[84-86]. During fetal and neonatal rat development cellular changes in receptor expression parallel detected levels in sera with peak levels expressed from 19 days gestation to 20 day old pups with dramatic decline thereafter[87]. Interestingly, Confort et. al. working with cultured MCF7 human breast cancer cells demonstrated that treatment with estrogen or IGF-II resulted in dramatic increase in both membrane bound and soluble CI-M6PR [48]. Currently no study correlates detectable serum or urine CI-M6PR levels either as a cancer marker or as an index for cancer progression. Though it is interesting to note that early reviews of biochemical markers in CSF for CNS tumours identify elevated levels of β-glucuronidase, a well recognized ligand of the CI-M6PR, in the presence of both primary and metastatic lesions[88, 89].

In this study, however, we were unable to identify a mutation of the cytoplasmic domain of the CI-M6PR in Ed 189 Bt cells despite the fact that such a finding would have nicely explained both the membrane predilection of the receptor in these cells as well as the functional observations.

We also investigated the expression of the CI-M6PR in immature and mature CD8+ lymphocytes and DCs. No differences in surface expression were found between the mature and immature states of these cells. Furthermore, neither immature nor mature cells were vulnerable to GrB + AD killing. These findings suggest that GrB and the CI-M6PR do not play a role in activated T-cell or DC elimination.

The relevance of performing this study was two fold. First, CI-M6PR expression in these cells comparing immature and mature states has not previously been reported. Second, as was detailed in the introduction, melanomas and gliomas have developed many methods of avoiding immune surveillance and effector cell actions. In a study using human peripheral T-lymphocytes, Ikushima et. al. demonstrated that

CD26, a key molecule for activation of T-cells, co-localizes with the CI-M6PR and causes phosphorylation of CD26. Furthermore, internalization of CD26 and subsequent activation of T-cells under the influence of PHA could be blocked by M6P[90]. It is not currently known if CI-M6PR or CD26 expression is in any way altered in TILs or circulating peripheral lymphocytes in cancer patients. In a previous investigation, conditioned media from glioblastoma cells known to contain secreted TGFβ, was shown to inhibit IL-2 secretion by TILs[91]. It is conceivable therefore, that in tumours that overexpress and subsequently shed CI-M6PR from their membranes, the soluble receptor could interact with T-cells via CD26 and interrupt the normal bound CI-M6PR phosphorylation and internalization required for immune effector activation. Further studies are required to resolve this important issue.

In summary, we have investigated a number of gliomas and melanomas and found surface expression of the CI-M6PR to be highly variable. We have also determined that in one glioma, Ed 189 Bt, elevated surface expression was correlated with a complete inability to internalize GrB and insensitivity to GrB or CTL mediated killing. In contrast to findings by others that a mutation involving the transmenbrane and cytoplasmic tail regions in other tumours that could plausibly explain the findings of both elevated surface expression and defective function of the CI-M6PR, we did not find such a mutation. The search for such a mutation in other melanoma and glioma tumours and studies regarding the involvement of transport or co-transport will form the basis of ongoing investigation.

Future Directions:

1. Evaluate our tumour bank to determine the prevalence of LOH for the CI-M6PR in various grades of astrocytoma and melanoma.

2. Evaluate Ed 189 Bt conditioned culture media for the presence of truncated secreted/cleaved CI-M6PR.

3. Evaluate the effects of Ed 189 Bt conditioned media on T lymphocyte activation under the influence of PHA.

4. Evaluate blood and CSF of patients with high grade gliomas and melanomas for the presence of truncated secreted/cleaved CI-M6PR.

5. Explore the expression and influence of molecules such as PI-9 on the functional quality of the CI-M6PR in various explant tumour cell lines.

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